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**13. ABSTRACT** ***(Maximum 200 Words)***

This is the final report of DAMD-17-91-850-9. The project was completed and the original hypothesis that fluid flow sensitivity of bone cells decreases as a function of age was confirmed. Additionaly, several other interesting findings regarding mechanotransduction in bone were discovered. The main findings of the project were that that GJIC modulates the PGE2 response of bone cells to oscillating fluid flow. Interestingly, we found that $PGE_2$ release in response to oscillating flow does not appear to involve intracellular calcium. Additionally, we have established that while the $[Ca^{2+}]_i$ response to fluid flow does decrease as a function of age, GJIC does not. We also demonstrated that a lack of the purinergic receptor P2Y2 is at least partially responsible for the inability of fluid flow to mobilize $Ca^{2+}$ in some bone cell lines. In summary, the completion of the project has led to a greater understanding of bone cell mechanotransduction and how it changes as a function of age.

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## Introduction

Bone cells are normally found in voids in the mineralized matrix known as lacunae. Small tubes in the matrix, known as canaliculi, interconnect the lacunae and are occupied by cellular processes. Gap junctions form where the processes of neighboring cells come into contact allowing for cell-cell communication via signaling substances. The extracellular space between the cell membrane and the mineralized matrix is filled with fluid which communicates with the bone's vascular supply. As the bone matrix is cyclically loaded due to physical activity, fluid flows in the lacunar-canalicular network from regions of high matrix strain to regions of low matrix strain and back again. Physiologic levels of this oscillating fluid flow have been shown to be a potent stimulator of bone cells *in vitro*. Additionally, we have shown that flow induced signals are transmitted from cell to cell such that cells that are not responding to flow directly can indirectly respond to flow-induced signals transmitted via gap junctions from adjacent cells. The consequence of this is that an ensemble of cells coupled via gap junctions is more sensitive to the effects of fluid flow than an equivalent collection of uncoupled cells. Our central hypothesis is that *there is an age-related decrease in the cellular responsiveness to fluid flow which is compounded by a decrease in cell-cell communication.*

In the first year of funding we made the following key findings:

- Discovered that neither ROS cells nor the RCx16 and ROS/Cx45 transfectants of ROS cells exhibit increase $[Ca^{2+}]_i$ in response to oscillating fluid flow.
- Discovered that ROS cells as well as the RCx16 and ROS/Cx45 transfectants of ROS cells release PGE2 in response to oscillating fluid flow.
- Accumulated early evidence that cell-cell communication via gap junctions plays an important role in regulating bone cell anabolism.

In the second year of funding we made the following key findings:

- Discovered that RCx16 and ROS/Cx45 gap junction deficient cells are less responsive to oscillatory fluid flow in terms of PGE2 release than ROS cells.
- Discovered that MC3T3-E1 cells exhibit an increase $[Ca^{2+}]_i$ in response to oscillating fluid flow, regardless of gap junctional intercellular communication (GJIC).
- Discovered that MC3T3-E1 cells release PGE2 in response to oscillating fluid flow via a mechanism that involves GJIC.
- Confirmed that the PGE2 response to oscillating fluid flow does not require a $[Ca^{2+}]_i$ response.
- Demonstrated that cell-cell communication via gap junctions plays an important role in regulating bone cell anabolism.
- Discovered that confluent and subconfluent cultures of rat osteoblastic cells have functional GJIC that is not age dependent.
- Discovered that the percentage of cells displaying fluid flow induced intracellular calcium signaling in ensembles of rat osteoblastic cells was significantly greater in cells from mature rats (12 mos.) than in cells from old rats (24 mos.) for an array of functional loading regimes.

In the third year of funding we made the following key findings:

- Confirmed that GJIC contributes to bone cell PGE2 but not $Ca^{2+}_i$ response to fluid flow. This was demonstrated in two different osteoblastic cell lines using several different techniques to inhibit GJIC.
- Confirmed that ROS cells do not display an increase in $[Ca^{2+}]_i$ in response to fluid flow.
- Discovered that the lack of a $Ca^{2+}$ response to fluid flow in ROS may be due to lack of purinergic P2Y2 receptors.

- Discovered that ATP interaction with purinergic receptors contributes to fluid flow responsiveness in both ROS and MC3T3-E1 cells.
- Discovered that there is a refractory period for fluid flow induced calcium mobilization in osteoblastic cells.

**Body**

**Aim 1:** (Year 1) Demonstrate that the responsiveness of a cell ensemble is related to the degree of cell-cell communication. Initially we proposed to examine this aim using three immortalized cell lines ROS (rat osteosarcoma), RCx16, and ROS/Cx45. Both RCx16 and ROS/Cx45 cells are ROS cells that have been transfected (but following different strategies) to limit GJIC. Previously we have shown that RCx16 and ROS/Cx45 cells are less coupled than ROS cells. We found that none of the ROS lines responded to either steady or oscillatory fluid flow with an increase in $[Ca^{2+}]_i$ This was true at several different flow rates and with perfusate media containing 2%, 5%, or 10% fetal bovine serum. However, ROS cells did respond to oscillatory fluid flow with an increase in PGE2 accumulation. Because the ROS cells do not demonstrate an increase in $Ca^{2+}$ in response to fluid flow we were unable to determine the role of GJIC in this process. To address this issue we completed experiments utilizing MC3T3-E1 cells expressing a dominant/negative Cx43. Wild type MC3T3-E1 cells responded to fluid flow with an increase in $Ca^{2+}$ and are thus an appropriate model to examine the role of GJIC in the $Ca^{2+}$ response to fluid flow.

The detailed results of our MC3T3-E1 experiments are contained in the published manuscript Saunders et al American Journal of Physiology: Cell 281:C1917-1925, 2001 (appendix 1). In summary we demonstrated that GJIC assessed by dye diffusion is diminished in the genetically manipulated cell line (DN-8) after 96 hours in culture, but not prior at 48 and 24 hours in culture. The control and DN-8 cells exhibited equivalent $[Ca^{2+}]_i$ responsiveness to flow, suggesting that $[Ca^{2+}]_i$ responsiveness to flow does not involve GJIC. In contrast, we found dramatically diminished release of PGE2 in response to flow in the coupling deficient DN-8 cells (96 hours) suggesting that the PGE2 response to flow does depend on GJIC.

These findings raised the possibility that the PGE2 response to flow does not involve a flow-induced increase in $[Ca^{2+}]_i$. To verify this interpretation we conducted an additional series of experiments using a blocker of intracellular cytosolic calcium mobilization. The results of these experiments demonstrate that PGE2 release in response to fluid flow can occur without a $[Ca^{2+}]_i$ increase. This conclusion is also consistent with our findings in the previous experiments utilizing ROS cells where we observed a PGE2 response to flow in a cell line that did not exhibit a $[Ca^{2+}]_i$ response to flow. This conclusion is highly significant since it suggests that the fluid flow response (and specifically that to oscillatory fluid flow) is unique in that PGE2 release can occur without the $[Ca^{2+}]_i$ response, which has not been previously observed for other physical and hormonal stimuli.

Since ROS cells respond to fluid flow with an increase in PGE2, despite not responding to flow with an increase in $[Ca^{2+}]_i$, we were able to examine the role of GJIC in fluid flow induced PGE2 release. Using steady and oscillatory fluid flow, mechanical stimulation was applied to ROS cells and a derivative of these cells expressing antisense cDNA for the gap junction protein connexin 43 (RCx16) which possess significantly different levels of GJIC and quantified $PGE_2$ and $Ca^{2+}$ responses. We found that osteoblastic cells possessing increased GJIC also exhibited increased $PGE_2$ accumulation in supernatant following fluid flow stimulation. These results, which are detailed in Saunders et al Bone 2003 (in press; appendix 2), confirm our previous results from MC3T3-E1 cells. Thus, using two different cell lines and two different genetic manipulations we were able to demonstrate that GJIC contributes to bone cell responsiveness to fluid flow.

We have also initiated experiments to examine the surprising finding that ROS cells do not respond to fluid flow with an increase in $Ca^{2+}$. Previous studies suggest that ROS cells do not express the purinergic receptor P2Y2. This receptor has been implicated in ATP regulation of $Ca^{2+}$ in bone cells. Furthermore, ATP has been implicated in the mechanism by which fluid flow increases $[Ca^{2+}]_i$ in endothelial cells. Therefor we examined the hypothesis that the lack of P2Y2 in ROS cells at least partially contributes to their inability to increase $[Ca^{2+}]_i$ in response to fluid flow. To examine this hypothesis we obtained ROS cells transfected with P2Y2 cDNA (ROS/P2U). We found that only 25.8% of wild type ROS 17/2.8 cells lacking P2Y2 receptors responded with an increase in $[Ca^{2+}]_i$ whereas 69.0% of ROS/P2U cells responded. This suggest that the P2Y2 receptor, and therefore ATP, contributes to fluid flow induced increases in $[Ca^{2+}]_i$ in ROS cells. To determine whether this is the case in other osteoblastic cells we exposed MC3T3-E1 cells to oscillating fluid flow in the presence of P2Y2 antisense oligonucleotides. We found that P2Y2 antisense oligonucleotide treatment decreased the percentage of cells responding to flow with an increase in $[Ca^{2+}]_i$, relative to scrambled P2Y2 oligonucleotide treatment (29.0% vs. 65.3% respectively). Taken together these results suggest that whereas GJIC does not appear to be critical for fluid flow induced increases in $[Ca^{2+}]_i$ a functional P2Y2 purinergic receptor is. This suggests that nucleotides such as ATP contribute to fluid flow responsiveness in bone cells. These results have been published in You et al, Journal of Biological Chemistry 277(50)48724-48729 (appendix 3)..

**Aim 2:** (Years 2 and 3) Quantify the responsiveness of bone cells as a function of age and cell-cell communication. Bone cells will be cultured from young, mature, and old rats and exposed to the fluid flow protocol of Aim 1. We have demonstrated that confluent cultures of rat osteoblastic cells (ROB) from young, mature, and old animals display highly functional GJIC. We found comparable functional communication in subconfluent ROB, from all three age groups, that were seeded on quartz microscope slides for calcium analysis.

Fluid flow induced shear stress was used as a mechanical stimulus to study intracellular calcium signaling in ROB that were isolated from young, mature, and old animals. Fura-2 was used to measure $[Ca^{2+}]_i$ in cells that were exposed to three minutes of oscillating fluid flow that produced shear stresses of 1 or 2 Pascals (Pa) at frequencies of 0.2, 1, or 2 Hz. Fluid flow caused an immediate and transient increase in $[Ca^{2+}]_i$. A significantly higher percentage of mature ROB displayed calcium transients than old ROB. Cells were more responsive to 0.2 Hz than to 1 or 2 Hz, and to 2 Pa than 1 Pa. These data suggest that intracellular calcium signaling is an important mechanotransduction response in rat osteoblastic cells and that there are age-related as well as frequency and shear stress amplitude dependent responses to oscillatory fluid flow. This work has recently been published in Donahue et al American Journal of Physiology: Cell 281:C1635-C1641, 2001 (appendix 4).

With support from this grant we have been able to examine other aspects of fluid flow effects on osteoblasts not directly related to our original hypothesis but with important implications regarding bone mechanotransduction. Previous studies suggest that bone cells exposed to mechanical signals display a refractory period, during which they are insensitive to additional mechanical stimuli. This may partially explain why intermittent loading is more anabolic than continuous loading. To examine the hypothesis that bone cells display a refractory period we exposed rat osteoblasts to sequential bouts of fluid flow separated by various time periods. We found that while a small percentage of cells could respond to a second bout of fluid flow after only a 5 second rest period, significantly ($p < 0.04$) fewer cells displayed $[Ca^{2+}]_i$ oscillations when the rest period was less than 600 seconds. Additionally, the magnitude of the second $[Ca^{2+}]_i$ oscillation was significantly ($p < 0.01$) lower than the magnitude of the first $[Ca^{2+}]_i$ oscillation for rest periods less than 900 seconds. As many as four fluid flow induced $[Ca^{2+}]_i$ oscillations could be invoked when rest periods of 2700 seconds were given between each bout. However, during one hour of continuous oscillating fluid flow, no subsequent $[Ca^{2+}]_i$ oscillations were observed after the initial immediate response. These findings suggest that a rest period is required for multiple fluid flow induced $[Ca^{2+}]_i$ responses in osteoblastic cells, but the refractory period may be as short as 5 seconds for some individual cells. However, a 900 second rest period was required to recover both the percentage of

cells responding and the magnitude of the response. *In vivo*, rest periods enhance mechanically induced bone formation. Therefore, it is reasonable to hypothesize that $[Ca^{2+}]_i$ oscillations play a role in *in vivo* bone adaptation. These results have been accepted for publication in the Journal of Biomechanics (appendix 5).

We have also examined the effect of fluid flow and GJIC in osteocytic cells. Cells were exposed for one hour to oscillating fluid flow at a shear stress of ± 10 dynes/cm$^2$ and a frequency of 1 Hz in a parallel plate flow chamber. Control cells were incubated in the chamber but were not exposed to oscillating fluid flow. Functional analysis of GJIC indicated that MLOY-4 cells exposed to oscillating fluid flow established more gap junctions with an independent population of dye labeled cells than did control cells. Phosphorylation of Cx43 was quantified by immunoprecipitation with an anti-Cx43 antibody followed by immunoblot analysis using an anti-phosphoserine antibody. Phosphoserine was normalized to Cx43 in each sample. Compared to control cells, phosphoserine content of Cx43 increased approximately two-fold in cells exposed to oscillating fluid flow. The possible role of the extracellular signal regulated kinase (ERK1/2) in the flow-induced upregulation of GJIC was also investigated. The ERK1/2 inhibitor PD-98059 significantly attenuated the effects of oscillating fluid flow on MLOY-4 cell GJIC. These results indicate that oscillating fluid flow regulates GJIC in MLOY-4 cells via the ERK1/2 MAP kinase. In addition, increased serine phosphorylation of Cx43 correlates with the flow-induced increase in GJIC. These results have been submitted for publication in the Journal of Bone and Mineral Research.

Because it has been hypothesized that bone cells have a hyaluronic acid (HA) rich glycocalyx that contributes to bone cell mechanotransduction we explored the role of the glycocalyx in bone cell response to fluid flow. The glycocalyx of bone cells of the MC3T3-E1 osteoblastic cell line and the MLO-Y4 osteocytic cell line were characterized. Alcian blue staining and lectin binding experiments suggested that these cells have a glycocalyx rich in HA. Sulphated proteoglycans were not detected. Staining with hyaluronic acid binding protein and degradation by hyaluronidase confirmed that HA was a major component of the glycocalyx. We subjected cells, with and without hyaluronidase treatment, to oscillating fluid flow under standardized *in vitro* conditions. There was no effect of glycocalyx degradation on the intracellular calcium signal, in either cell type in terms of the percentage of cells responding (40-80%) or the magnitude of the response (2-5 times baseline). However a 4-fold fluid flow induced increase in PGE2 was almost eliminated by hyaluronidase pre-treatment in MLO-Y4 cells. We conclude that under these conditions the calcium and PGE2 responses occur via different pathways. An intact glycocalyx is not necessary in order to achieve an intracellular calcium signal in response to oscillating fluid flow. However, in osteocyte-like cells the PGE2 pathway is more dependent on mechanical signals transmitted through the glycocalyx. These results have been submitted for publication in the Journal of Bone and Mineral Research.

We have also explored candidate mechanoreceptors in bone cells that may transduce mechanical signals, such as fluid flow, through the cell membrane. One such candidate is annexin (AnxV) a membrane channel that has previously been proposed as a good candidate for a mechanoreceptor. Therefore, we examined the role of AnxV in bone cell response to fluid flow. Oscillating fluid flow increased both $[Ca^{2+}]_i$ levels and cFOS protein levels in osteoblasts. Disruption of AnxV with specific blocking antibodies or a pharmacological inhibitor, K201 (JTV-519), significantly inhibited both responses. Additionally, our data demonstrated that oscillating fluid flow modulated the cellular location of AnxV. Exposure to oscillating fluid flow resulted in a significant increase in AnxV at both the plasma and nuclear membranes. Our data suggest that AnxV is involved in the mechanism by which mechanical signals are transduced into cellular responses in the osteoblast. These results have been submitted for publication in the Journal of Biological Chemistry.

Finally, we have begun to examine aspects of fluid flow other than shear stress that might affect bone cell activity. In addition to shear stress, loading-induced fluid flow will enhance chemotransport due to convection or mass transport thereby affecting the biochemical environment surrounding the cell.

Therefore, we investigated the role of oscillating fluid flow-induced shear stress and chemotransport in cellular mechanotransduction mechanisms in bone. Intracellular calcium mobilization and $PGE_2$ production were studied with varying shear stresses and chemotransport. MC3T3-E1 cells responded to oscillating fluid flow with both a robust increase of intracellular calcium concentration $[Ca^{2+}]_i$ and an increase in $PGE_2$ production. These fluid flow induced responses were modulated by chemotransport. The percentage of cells responding with an increase in $[Ca^{2+}]_i$ varied linearly with flow rate, as did the production of $PGE_2$. In addition, depriving the cells of nutrients during fluid flow resulted in an inhibition of intracellular calcium mobilization and the production of $PGE_2$. These data suggest that depriving the cells of a yet to be determined biochemical factor in media affects the responsiveness of bone cells even at a constant peak shear stress. Chemotransport alone will not elicit a response, but it appears that sufficient nutrient supply or waste removal is needed for the response to oscillating fluid flow induced shear stress. These results have been submitted for publication in the Journal of Biomechanics.

**Aim 3:** (Years 3 and 4) Examine the effect of forskolin on fluid flow responsiveness in osteoblasts as a function of age. This aim was designed to examine the ability of forskolin to ameliorate age-related decreases in GJIC and thereby restore bone cell sensitivity to fluid flow. However, since we found that there was not an age-related change in GJIC we chose not to pursue this aim. Instead we pursued the interesting areas outlined above.

**Key Research Accomplishments**

- Demonstrated that oscillating fluid flow regulates GJIC in osteocytic MLOY-4 cells via the ERK1/2 MAP kinase. In addition, increased serine phosphorylation of Cx43 correlates with the flow-induced increase in GJIC.
- Demonstrated that an intact glycocalyx is not necessary in order to achieve an intracellular calcium signal in response to oscillating fluid flow. However, in osteocyte-like cells the PGE2 pathway is more dependent on mechanical signals transmitted through the glycocalyx.
- Demonstrated that AnxV is involved in the mechanism by which mechanical signals are transduced into cellular responses in the osteoblast.
- Demonstrated that both shear stress and chemotransport contribute to bone cells response to fluid flow.

**Reportable Outcomes**

The following manuscripts and abstracts were supported either partially or fully by the project:


1. Yellowley, C.E., Jacobs, C.R. and Donahue, H.J. (1999) Mechanisms contributing to fluid-flow-induced $Ca^{2+}$ mobilization in articular chondrocytes. Journal of Cellular Physiology. 180(3):402-8.

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15. Saunders, M.M., You, J., Zhou, Z., Li, Z., Yellowley, C.E., Kunze, E.L., Jacobs, C.R. and Donahue, H.J. (2003) Fluid flow-induced prostaglandin $E_2$ response of osteoblastic ROS 17/2.8 cells is gap junction-mediated and independent of cytosolic calcium. Bone (in press).

16. Alford, A.I., Jacobs, C.R., Donahue, H.J. (2003) Oscillating fluid flow regulates gap junction communication in osteocytic MLO-Y4 cells by an ERK1/2 MAP kinase dependent mechanism. J. Bone Min. Res. (in review).

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Abstracts

1. Saunders, M.M., You, J., Yellowley, C.E., Jacobs, C.R. and Donahue, H.J. (2000) Differential effect of oscillating flow on cytosolic calcium and prostaglandin in osteoblastic ROS 17/2.8 cells. 46$^{th}$ Annual Meeting of the Orthopaedic Research Society.

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3. Edlich, M., Yellowley, C.E., Jacobs, C.R. and Donahue, H.J. (2000) Effect of fluid flow on cycle number and waveform on intracellular calcium concentration in chondrocytes. 46$^{th}$ Annual Meeting of the Orthopaedic Research Society.

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## Conclusions

We have successfully completed aims one and two of this project. For aim one we have demonstrated our hypothesis to be true, namely that GJIC modulates the PGE2 response of bone cells to oscillating fluid flow. Interestingly, we found that $PGE_2$ release in response to oscillating flow does not appear to involve intracellular calcium. This has important implications because it indicates that other second messengers, such as the cyclic AMP pathway, may be important in transducing the fluid flow signal into the $PGE_2$ response. Additionally, the differential effect of oscillating fluid flow on $[Ca^{2+}]_i$ versus $PGE_2$ may prove to be a powerful tool in further investigations of the bone cell mechanotransduction pathway. Additionally, as regards aim two, we have established that while the $[Ca^{2+}]_i$ response to fluid flow does decrease as a function of age, GJIC does not. Our success with the first two aims have led to other interesting findings. For instance, we have demonstrated that a lack of the purinergic receptor P2Y2 is at least partially responsible for the inability of fluid flow to mobilize $Ca^{2+}$ in ROS cells. This has led to our investigation of the role of ATP in mechanotransduction in bone. This is proving to be a very exciting area of research. Research supported by this funding has resulted in 15 publications and 5 manuscripts in review. The results have been incorporated into two applications to the NIH which are now in their third year, another R01 that is indirectly related and in its first year, an NIH Individual National Research Award in its first year and a Whitaker foundation grant in its first year. Thus, we believe that the project has not only led to a better understanding of mechanotransduction in bone but has also enabled us to greatly enhance our research effort in this area.

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# Gap junctions and fluid flow response in MC3T3-E1 cells

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**Saunders, M. M., J. You, J. E. Trosko, H. Yamasaki, Z. Li, H. J. Donahue, and C. R. Jacobs.** Gap junctions and fluid flow response in MC3T3-E1 cells. *Am J Physiol Cell Physiol* 281: C1917–C1925, 2001.—In the current study, we examined the role of gap junctions in oscillatory fluid flow-induced changes in intracellular $Ca^{2+}$ concentration and prostaglandin release in osteoblastic cells. This work was completed in MC3T3-E1 cells with intact gap junctional communication as well as in MC3T3-E1 cells rendered communication deficient through expression of a dominant-negative connexin. Our results demonstrate that MC3T3-E1 cells with intact gap junctions respond to oscillatory fluid flow with significant increases in prostaglandin $E_2$ ($PGE_2$) release, whereas cells with diminished gap junctional communication do not. Furthermore, we found that cytosolic $Ca^{2+}$ ($Ca_i^{2+}$) response was unaltered by the disruption in gap junctional communication and was not significantly different among the cell lines. Thus our results suggest that gap junctions contribute to the $PGE_2$ but not to the $Ca_i^{2+}$ response to oscillatory fluid flow. These findings implicate gap junctional intercellular communication (GJIC) in bone cell ensemble responsiveness to oscillatory fluid flow and suggest that gap junctions and GJIC play a pivotal role in mechanotransduction mechanisms in bone.

prostaglandin $E_2$; calcium; mechanotransduction; gap junctional intercellular communication

IT IS WIDELY ACCEPTED that bone adapts to its physical loading milieu by optimizing mass and mechanical performance in a process known as bone remodeling. In a nonpathological scenario, this process results in normal bone turnover whereby new bone formation is balanced by removal of existing bone. In a pathological scenario, this process results in an imbalance whereby net bone formation (osteopetrosis) or net bone loss (osteopenia) ensues. Although the effects of remodeling have been histologically observed, the exact cellular pathways by which it occurs are incompletely understood. To this end, researchers have recently begun to investigate mechanotransduction mechanisms (15, 17, 32) in an attempt to better uncover the elusive signal transduction pathways by which physical stimuli can affect cellular responses in bone. These studies have found that bone cells can respond to a wide variety of endogenously occurring signals including mechanical stretch (41), streaming potentials, chemotransport, electrical effects (2, 6, 26, 33), and fluid flow (8, 17, 28, 41). In the latter area of research, it has been hypothesized that the fluid flow through the lacunar-canalicular network is pivotal to bone cell responsiveness. Although several hypotheses have been proposed, many believe that the osteocytes in the canalicular spaces sense the fluid flow (1) and in turn signal the osteoblasts to form bone.

Although many accept the theory that osteoblast responsiveness to biophysical effects is linked to the osteocyte (1, 23), few have proposed a mechanism by which this may occur. We propose that bone cell responsiveness to fluid flow is aided by gap junctions that physically connect osteoblasts and osteocytes (39) as well as osteoblasts to other osteoblasts. In this scenario, we hypothesize that gap junctions not only enable osteocytes to transfer signals to osteoblasts but that the responsiveness of osteoblastic networks to the signal is amplified via gap junctions. By coupling the osteoblasts together, gap junctions enable the cells to respond in concert, resulting in a more robust response attained than if an equal number of individual cell responses was achieved. This is the focus of our current work.

Gap junctions are transmembrane protein channels that enable neighboring cells to physically link, thereby facilitating the rapid diffusion of small molecules and ions on the order of 1 kDa in a process known as gap junctional intercellular communication (GJIC). Gap junctions may be homospecific, uniting cells of the same type, or may be heterospecific, uniting cells of unlike type. With the exception of blood cells and muscle fibers, gap junctions have been found in most cells (30), with at least 13 mammalian connexins identified to date and named with respect to molecular weight.

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There is growing evidence to support a role for gap junctions in the cellular (and cell ensemble) response to physical stimuli. In bone, gap junctions have been linked to such functions as hormonal responsiveness (34), gene expression (25), and differentiation (7). Intercellularly, gap junctions have been linked to second messenger responses induced by physical stimuli such as $Ca^{2+}$ release following membrane deformation (21). Interestingly, many fluid flow studies have shown that osteoblastic cells respond to flow in vitro with an increase in such second messengers as $Ca^{2+}$ (13, 15), cAMP (28), and NO release (20), which have been shown to be physical regulators of gap junction channel opening.

GJIC has been linked to both normal and abnormal cell function. In normal cell function, GJIC has been linked to such processes as proliferation and differentiation, although the findings at times have been inconsistent. For instance, while GJIC is found to maintain cell differentiation status in cultured hepatocytes (40), it is decreased in differentiating keratinocytes compared with proliferating ones (12). Furthermore, we have recently demonstrated that gap junctional function and expression parallel osteoblastic differentiation, contributing to alkaline phosphatase expression (7). Thus gap junction studies are widely dependent on cell line, culture conditions, and experimental environment, and results must be interpreted within these contexts. In abnormal cell function, alterations in GJIC have been linked to disease (30, 36), suggesting that a status quo in gap junctional function is crucial to homeostasis.

In the current study, we set out to examine the role of GJIC in transducing a mechanical stimulus to bone cells. That is, we exposed osteoblastic cells to levels of oscillatory fluid flow that occur in vivo due to habitual loading (35), and we measured prostaglandin $E_2$ ($PGE_2$) release and cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_i$), markers selected for their proposed role in the regulation of bone turnover (4, 10, 16, 19, 27). To correlate these findings with the role of GJIC, we utilized osteoblastic MC3T3-E1 cells, MC3T3-E1 cells expressing a dominant-negative connexin43 (Cx43; DN-8), the predominant gap junction protein in bone, and a control transfectant (DN-VC). Comparisons of changes in $PGE_2$ release and $[Ca^{2+}]_i$ in the presence of oscillatory fluid flow in the three cell lines were then used to draw conclusions about the contribution of gap junctions and GJIC to bone remodeling.

## MATERIALS AND METHODS

*Cell culture.* Three immortalized osteoblastic cell lines were utilized in this study: MC3T3-E1, DN-VC, and DN-8. The MC3T3-E1 is an immortalized mouse osteoblastic cell line; the DN-8 is a neomycin-sustained transfectant of MC3T3-E1 containing a mutant Cx43; and the DN-VC is a control for the transfection containing an empty plasmid. MC3T3-E1 cells were cultured in Eagles's minimal essential medium (MEM-α; GIBCO BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 1% penicillin/streptomycin (GIBCO BRL). The DN-8 and DN-VC cells were cultured in MC3T3-E1 medium supplemented with neomycin (200 μg/ml). All cell lines were maintained in an incubator at 37°C and 5% $CO_2$, with flow experiments conducted in the appropriate media supplemented with 2% FBS.

The DN-8 line was developed from a dominant-negative strategy as previously described (24). Briefly, a mutant gap junction protein (Cx43Δ) of Cx43 was developed by the deletion of residues in the internal cytoplasmic loop of the connexin structure. The goal of this strategy was to introduce this mutant gene into both protein channels of each linking cell such that the mutant could oligomerize with only a wild-type species. Unlike previous dominant-negative strategies in which GJIC is obliterated and the resulting connexin oligomers are not transported to the membrane but remain in the cytoplasm, this novel mutation approach affects only permeability, leaving transport intact (24).

*Cell preparation.* Experiments were conducted on two differently sized microscope slides. For the quantification of oscillatory fluid flow-induced cytosolic $Ca^{2+}$ ($Ca_i^{2+}$) mobilization, cells were plated on quartz slides (76 mm × 26 mm × 1.6 mm) for imaging. These slides accommodated the relatively few cells needed to conduct the experiments and were made of quartz to allow for ultraviolet visualization. Cells were plated at $1.0 \times 10^5$, $0.75 \times 10^5$, or $0.5 \times 10^5$ cells/slide and cultured for 24, 48, or 96 h, respectively, to achieve 85–90% confluence. For the quantification of oscillatory fluid flow-induced $PGE_2$ production, cells were plated on glass microscope slides (75 mm × 38 mm × 1 mm). These slides were larger so that larger volumes of cells could be evaluated. Cells were plated at $3.5 \times 10^5$, $2.75 \times 10^5$, or $2.0 \times 10^5$ cells/slide and cultured for 24, 48, or 96 h, respectively, to achieve 85–90% confluence. For quantification of GJIC, cells were plated as described for the $PGE_2$ experiments. In addition, cells for double labeling were cultured in round (35-mm-diameter) polystyrene petri dishes in the appropriate media for 24, 48, or 96 h.

*GJIC assays.* GJIC assays were completed using epifluorescent microscopy and a double-labeling technique, as previously described (39). In this technique, cells are loaded with the fluorescent dyes calcein AM (Molecular Probes, Eugene, OR) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes). The fluorescent dye calcein AM, once in the cell, is cleaved of its AM group and trapped within the cell. However, as a result of its small molecular size (<1 kDa), calcein is gap junction permeable and able to transfer to neighboring cells if functional (open) gap junctions are established. The fluorescent dye DiI is of a larger molecular size, intercalates within cell membranes, and does not transfer to neighboring cells via GJIC. The loaded cells are then dropped onto unloaded cells in a monolayer, and cell transfer is quantified. If functional gap junctions are established, the calcein will transfer to neighboring cells that will then fluoresce green.

Coupling assays were completed to establish the extent of disruption of GJIC in DN-8 cells at 24, 48, and 96 h in culture and were compared with GJIC in the MC3T3-E1 and DN-VC lines at the same time points. After quantification of GJIC, we assessed GJIC in the three cell lines at 96 h in culture simultaneously with $PGE_2$ and $[Ca^{2+}]_i$ experiments to minimize passage variables. On the day of the experiments, the preconfluent cells ("donor" cells) plated in the petri dishes were removed from the incubator and washed twice with room temperature phosphate-buffered saline (PBS) followed by aspiration. The donor cells were labeled with a BSA-enriched PBS-fluorescent dye mixture containing 20 μl of calcein AM, 7 μl of DiI, and 20 μl of pluronic acid (Molecular

Probes) and incubated for 30 min at 37°C. After being incubated, the dye mixture was aspirated, and the donor cells were washed twice in room temperature PBS. The donor cells were detached from the dishes by trypsinization, centrifuged at 200 *g* for 8 min, and resuspended in fresh growth medium. The double-labeled (calcein and DiI) donor cells were then dropped onto the glass slides containing confluent monolayers of unlabeled cells at a ratio of ~1:500 cells (labeled to unlabeled) and incubated for 90 min at 37°C. After the incubation period, the slides were removed from the dishes, washed twice with PBS, and covered by round (25-mm-diameter) glass coverslips. The slides were placed on a Nikon fluorescent microscope (Nikon EFD-3; Optical Apparatus, Ardmore, PA) and visualized using fluorescein ($\lambda_{excitation}$ = 465–495 nm; $\lambda_{emission}$ = 520 nm) and rhodamine ($\lambda_{excitation}$ = 541–551 nm; $\lambda_{emission}$ = 590 nm) filters to locate the calcein- and DiI-loaded cells, respectively. Coupling was quantified by counting the number of neighboring cells fluorescing green, while the DiI was used to distinguish the labeled cells from those in the monolayer. Thirty cells were randomly selected and counted for each slide. Coupling was considered extensive if individual cells transferred calcein to >15 cells and were not counted past this threshold number.

*Parallel plate flow chambers and testing machine.* For $PGE_2$ and $Ca^{2+}$ experiments, bone cells were placed in a parallel plate flow chamber and subjected to oscillatory fluid flow. This system has been previously characterized, and we and others have employed it to expose endothelial cells (8), chondrocytes (37, 38), and bone cells (14, 15, 17, 41) to physiological levels of fluid flow. Briefly, the system imparts a laminar flow to the cells in a monolayer, exposing them to a shear stress governed by the equation (9)

$$\tau = 6\mu Q/bh^2$$

where $\tau$ is the shear stress, $\mu$ is the viscosity of the flow medium, Q is the flow rate, and $b$ and $h$ are the width and height of the chamber, respectively. To accommodate the quartz and glass microscope slides, as previously noted, two differently sized chambers were employed. In general, the components for both chambers were the same and are shown in the exploded view of Fig. 1. The chambers consisted of a polycarbonate manifold, a silastic gasket, and a glass slide. This slide containing the cells in a monolayer formed the bottom of the flow chamber when inverted on the manifold. For $Ca^{2+}$ studies, an 18 ml/min flow rate resulted in a shear

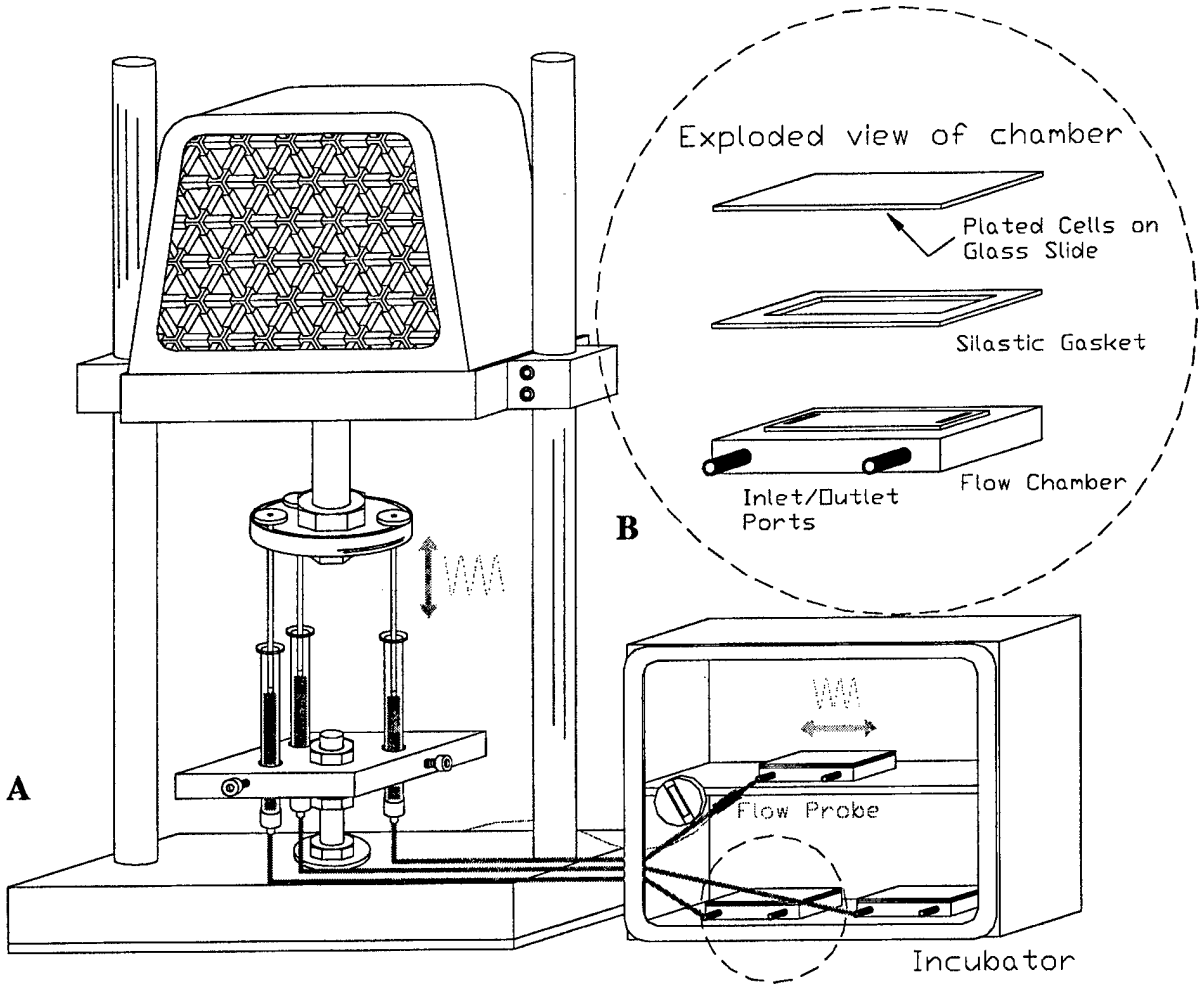


Fig. 1. Schematic of oscillatory fluid flow delivery system. *A*: oscillatory fluid flow was delivered via a sinusoidal waveform generated by a materials testing machine connected to the flow chamber using tubing and syringes. *B*: the flow chamber consisted of a parallel plate design. Cells in a monolayer on the glass slide were inverted on the flow chamber on a silastic gasket. During fluid flow, the assembly was held together with either a vacuum seal or encased in a polycarbonate case (not shown).

stress of 20 dyn/cm$^2$ and a rectangular flow volume of 38 mm × 10 mm × .28 mm; for $PGE_2$ studies, a 43 ml/min flow rate resulted in a shear stress of 20 dyn/cm$^2$ and a rectangular flow volume of 56 mm × 24 mm × 0.28 mm. In all flow experiments, flow rate was monitored with an ultrasonic flow probe (Transonic Systems, Ithaca, NY) connected to the chamber inlet. For $Ca^{2+}$ imaging, the flow chamber assembly was held together with vacuum pressure; for $PGE_2$ quantification, the flow chamber assembly was placed in a polycarbonate case bolted together to form an air-tight seal. For the latter experiments, the polycarbonate case containing the chamber enabled the system to be placed in an incubator for long-term flow periods (1 h) such that temperature and $CO_2$ levels could be precisely regulated. For both short- ($Ca^{2+}$) and long-term ($PGE_2$) experiments, the chamber was connected to a pneumatic, closed-loop feedback materials testing machine (EnduraTec, Minnetonka, MN) via tubing and syringes with oscillatory fluid flow delivered in the form of a 1-Hz sine wave (Fig. 1).

*$PGE_2$ quantification.* $PGE_2$ accumulation in the supernatant was quantified with a commercially available, nonradioactive, competitive binding enzyme immunoassay system (BioTrak; Amersham Pharmaceuticals, Piscataway, NJ). After assay, the optical densities of the samples were read at 450 nm using a microplate reader (Dynex Technologies, Chantilly, VA). Manufacturer-supplied standards were also analyzed and used to construct a standard curve from which the sample concentrations were determined.

Oscillatory fluid flow-induced $PGE_2$ was quantified at the 48- and 96-h time points. On the day of the experiments, preconfluent slides of cells were washed, placed in the parallel plate flow chamber, encased in the polycarbonate case, placed in the incubator, and connected to the fluid flow delivery system. Cells were exposed to flow for 1 h, after which 10 ml of media from the inlet and outlet ports of the chamber and adjacent tubing were collected for $PGE_2$ analysis. These media are referred to throughout as media collected immediately postflow. In addition, the plates of cells were incubated in 10 ml of fresh medium for 1 h postflow, and these media were also collected for $PGE_2$ analysis. These media are referred to throughout as media collected 1 h postflow. Immediately after media collection, aliquots were frozen at −80°C. In addition, for some experiments, the ionophore 4-bromo-calcium (50 μM) was added to a plated slide from each cell line for 15 min at 37°C. The media from these collections were used as positive controls in the $PGE_2$ assays. On the day of assay, samples were thawed at 4°C and vortexed. Assays were completed at room temperature within 1 mo of collection, and degradation assays were completed to ensure that this time period did not adversely affect the results.

The three cell lines were also subjected to oscillatory flow in the presence of thapsigargin, a drug used in our study to empty and prevent refilling of intracellular $Ca^{2+}$ stores, thus eliminating this source of $Ca^{2+}$ contributing to changes in $[Ca^{2+}]_i$ (42). $PGE_2$ experiments in the presence of thapsigargin were completed at the 96-h time point following the exact protocol previously outlined with one exception: thapsigargin (50 nM) was added to each petri dish of plated cells (30 min before placing it in the flow chambers), the flow medium, and the 10 ml of fresh, 1-h postflow incubation medium.

Because total $PGE_2$ accumulation in the medium is dependent on cell number, prostaglandin accumulation was normalized to total cell protein for each slide. After the 1-h incubation and collection of the additional 10 ml of fresh medium, the cells were removed from each microscope slide by trypsinization, centrifuged at 200 *g* for 8 min, and resuspended in 0.5 ml of 0.05% Triton X-100 detergent. The suspended cells were placed in 1-ml centrifuge tubes and lysed using three cycles of rapid freezing (−80°C) and thawing. The lysate was frozen at −80°C until analysis with a commercially available assay kit (Bio-Rad, Hercules, CA). After assay, the optical densities of the samples were read at 405 nm using a microplate reader. Manufacturer-supplied standards were analyzed and used to construct the standard curve from which the sample concentrations were determined. Frozen cells and media were assayed at room temperature within 1 mo of collection.

*$Ca^{2+}$ imaging.* $Ca^{2+}$ imaging was completed with fluorescent microscopy and the dual-wavelenth ratiometric dye fura 2-AM (Molecular Probes). This indicator was selected for its ability to exhibit two distinct spectra and two distinct wavelengths based on the presence or absence of $Ca^{2+}$ binding to the indicator (31). The indicator is loaded in the fura 2-AM form, which allows it to easily enter the cells. After loading, the AM groups are cleaved in an enzymatic process leaving the indicator trapped within the cell.

$[Ca^{2+}]_i$ was quantified in the three cell lines at the 96-h time point. On the day of the experiments, preconfluent slides of cells were loaded with 10 μM fura 2-AM in 1 ml of fresh media and incubated at 37°C for 45 min. After being incubated, the cells were washed in the appropriate flow medium (2% FBS), placed on the parallel plate flow chamber, transferred to a fluorescent microscope, and connected to the loading machine. To allow the cells to settle and ensure that the AM hydrolizing process was complete, the cells were allowed to equilibrate on the microscope stage for 30 min immediately before testing. Cells were subjected to 3 min of oscillatory flow preceded by a 3-min no-flow baseline. An image acquisition and analysis software package (Metafluor) was used to capture the images for $[Ca^{2+}]_i$ determination.

*Data analyses.* $Ca^{2+}$ results were analyzed with a Rainflow counting technique (18). This technique, adapted from the field of mechanical fatigue, enables individual responses to be extracted from data containing multiple responses and has generally been employed to determine the contribution of a particular loading cycle to the overall lifetime of a structure. Rainflow applied to our research enabled individual cell responses to be isolated and separated from background noise with a threshold response defined as a change in $[Ca^{2+}]_i$ of ≥20 nM.

$PGE_2$ results were analyzed using a microplate reader and normalized to total protein with total $PGE_2$ accumulation in the medium given in picograms per micrograms. GJIC was quantified by counting cell fluorescence transfers, as previously described. All $Ca^{2+}$, $PGE_2$, and GJIC data obtained were expressed as means ± SE. To compare results among the cell lines, general linear model ANOVAs with Student-Newman-Keuls post hoc comparisons were completed using a commercially available software program (Instat; GraphPad Software, San Diego, CA) with an a priori significance level of 0.05.

## RESULTS

*Osteoblastic cell line GJIC as a function of time in culture.* GJIC was qualitatively evaluated at 24-, 48-, and 96-h time points in the three cell lines with typical dye transfers shown (Fig. 2). In these double-exposed photographs, the green (calcein) fluorescence indicates the coupled cells in the monolayer, whereas the yellow (calcein and DiI) fluorescence indicates double-labeled donor cells. Quantitative results for the 24-, 48-, and

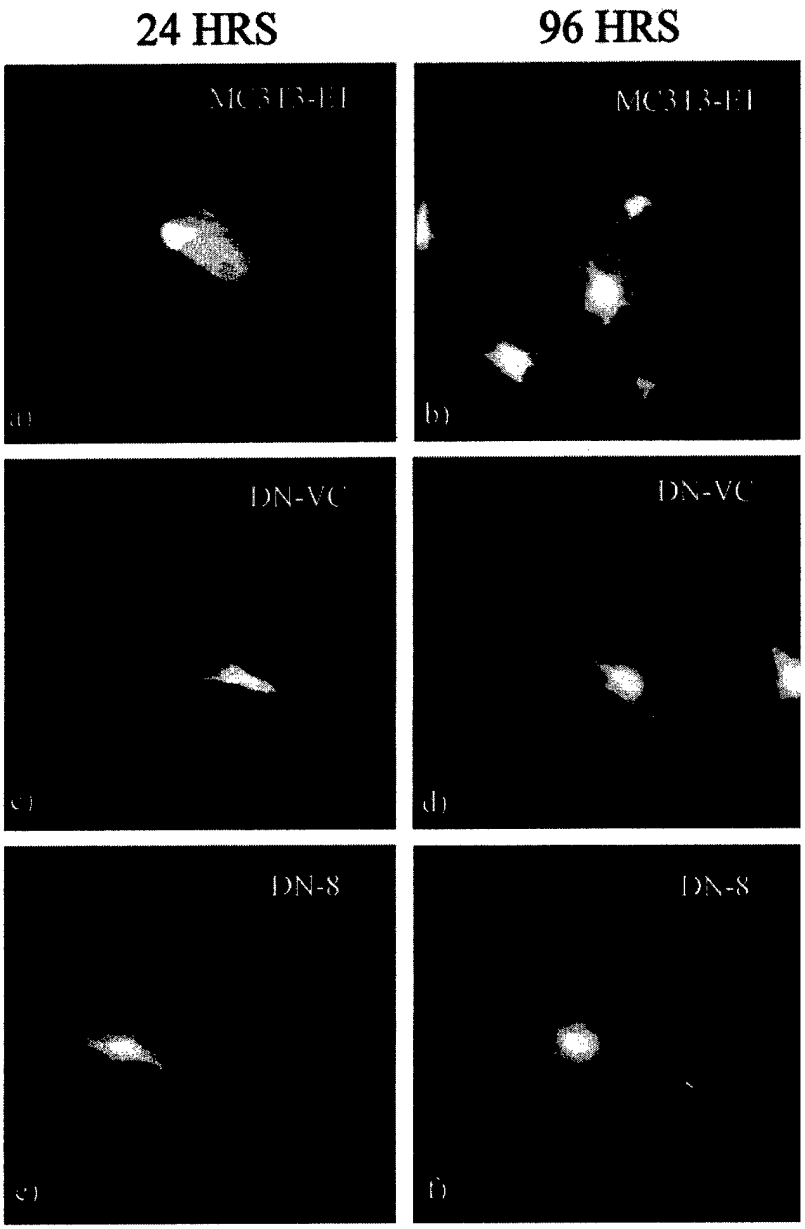


Fig. 2. Qualitative results of double-labeling assay at 24 and 96 h in the 3 cell lines examined. MC3T3-E1 (*A* and *B*), DN-VC (*C* and *D*), or DN-8 (*E* and *F*) cells were grown in the monolayer for 24, 48 (not shown), or 96 h and subjected to homospecific gap junctional intercellular communication analysis. Donor cells double labeled with the fluorescent dyes calcein and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) were placed in contact with unloaded like cells in the monolayer. Cell transfer was visualized after 90 min. In the dual-exposure photographs, the cells fluorescing green (calcein) are the unlabeled cells in the monolayer demonstrating functional coupling; the cells fluorescing yellow (calcein and DiI) are the dual-labeled donor cells (original magnification, ×400).

96-h time points (Fig. 3) depict the number of donor cells coupled to individual acceptor cells in the monolayer. We found that the MC3T3-E1 and DN-VC cell line coupling was not dependent on time in culture up to 96 h. At 24 and 48 h, the three cell lines did not exhibit a significant difference in coupling compared with each other. However, at the 96-h time point, DN-8 cells exhibited a significant decrease in coupling compared with the MC3T3-E1 ($P < 0.001$) and DN-VC ($P < 0.001$) lines at 96 h, as well as compared with themselves at the 24- ($P < 0.001$) and 48-h ($P < 0.001$) time points.

*$PGE_2$ accumulation in response to fluid flow.* Because GJIC was decreased in DN-8 cells only after 96 h in culture, we first examined $PGE_2$ response to fluid flow at this time point (Fig. 4*A*). Media from MC3T3-E1 and DN-VC cells collected 1 h postflow accumulated significantly more $PGE_2$ than cells not exposed to flow ($P < 0.0005$ and $P < 0.0001$, respectively). However, media from poorly coupled DN-8 cells did not accumulate more $PGE_2$ than control cells. Similar results were obtained when media were collected immediately postflow (not shown).

We also examined the effect of fluid flow on $PGE_2$ accumulation in DN-8 cells cultured at 48 h, a period after which DN-8 cells are as well coupled as MC3T3-E1 cells (Fig. 4*B*). Whereas exposure to fluid flow did not increase $PGE_2$ accumulation in media collected 1 h postflow from DN-8 cells cultured for 96 h, it did increase in media from DN-8 cells cultured for 48 h ($P < 0.005$ vs. no-flow controls). Similar results were obtained when media were collected immediately postflow (data not shown).

*$Ca^{2+}$ response to oscillatory fluid flow.* In cells cultured for 96 h, there was a 7.9-fold increase ($P < 0.0007$

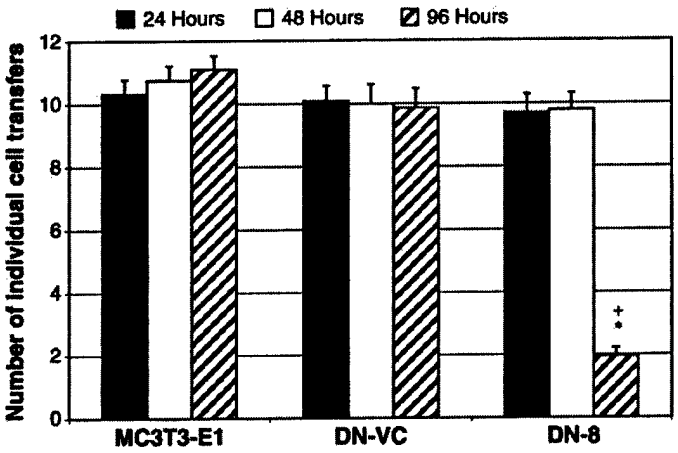


Fig. 3. Quantitative results of double-labeling assay at 24, 48, and 96 h in the 3 cell lines examined. MC3T3-E1 and DN-VC cell lines were highly coupled at all time points. No significant differences in coupling were found within or between these 2 cell lines at the various time points. The DN-8 cell line was well coupled at 24 and 48 h and not significantly different from the other cell lines at these time points. At 96 h, coupling in the DN-8 line was significantly diminished compared with the DN-8 line at 24 and 48 h ($P < 0.001$) as well as compared with the MC3T3-E1 and DN-VC lines at this time point ($P < 0.001$). Each bar is representative of a minimum of 60 cells (maximum 110) and is plotted as means ± SE with individual cell transfers not counted past a maximum of 15 cells. *Significantly different from 24- and 48-h time points within group; $^+$significantly different from 96-h time points in MC3T3-E1 and DN-VC cell lines.

vs. no flow) in the percentage of MC3T3-E1 cells responding to 3 min of oscillatory fluid flow with an increase in $[Ca^{2+}]_i$, an 8.9-fold increase in DN-VC cells ($P < 0.0001$), and a 9.3-fold increase in DN-8 cells ($P < 0.0003$; Fig. 5). The fold increases were not statistically different among the three cell lines. No significant differences in $[Ca^{2+}]_i$ amplitude within or among groups were observed (data not shown), a finding also made in our previous work with human fetal osteoblastic cells (41).

*$PGE_2$ accumulation in the presence of thapsigargin.* One interpretation of our findings that GJIC contributed to the $PGE_2$ but not the $[Ca^{2+}]_i$ response to fluid flow in DN-8 cells is that $Ca_i^{2+}$ mobilization may not be critical to fluid flow-induced $PGE_2$ accumulation. To address this issue, we examined the effect of thapsigargin on fluid flow-induced $PGE_2$ accumulation. In the presence of thapsigargin, media from MC3T3-E1 and DN-VC cells collected 1 h postflow had a 92.1% and 278%, respectively, increase in $PGE_2$ accumulation relative to no-flow control. Once again, fluid flow did not increase $PGE_2$ accumulation in DN-8 cells cultured for 96 h and thus coupled poorly. Therefore, thapsigargin did not significantly alter the $PGE_2$ response to fluid flow in any of the cell lines examined (Fig. 6).

## DISCUSSION

In this study, we set out to investigate the role of gap junctions and GJIC in mechanotransduction mechanisms in bone. We applied a novel dominant-negative genetic intervention strategy to MC3T3-E1 osteoblastic parent cells to render them communication deficient. We subjected the resulting cell line to oscillatory fluid flow and measured flow-induced $PGE_2$ release and changes in $[Ca^{2+}]_i$. This is the first study to examine GJIC in bone cell ensemble responsiveness to fluid flow, and, while only the second study to examine the effects of oscillatory fluid flow on $[Ca^{2+}]_i$ in osteoblastic cells, it is the first to quantify the $PGE_2$ response. We found that a breakdown in gap junction coupling had no effect on changes in $[Ca^{2+}]_i$ but resulted in a significant inhibition of oscillatory fluid flow-induced $PGE_2$ release, suggesting that gap junctions play a pivotal

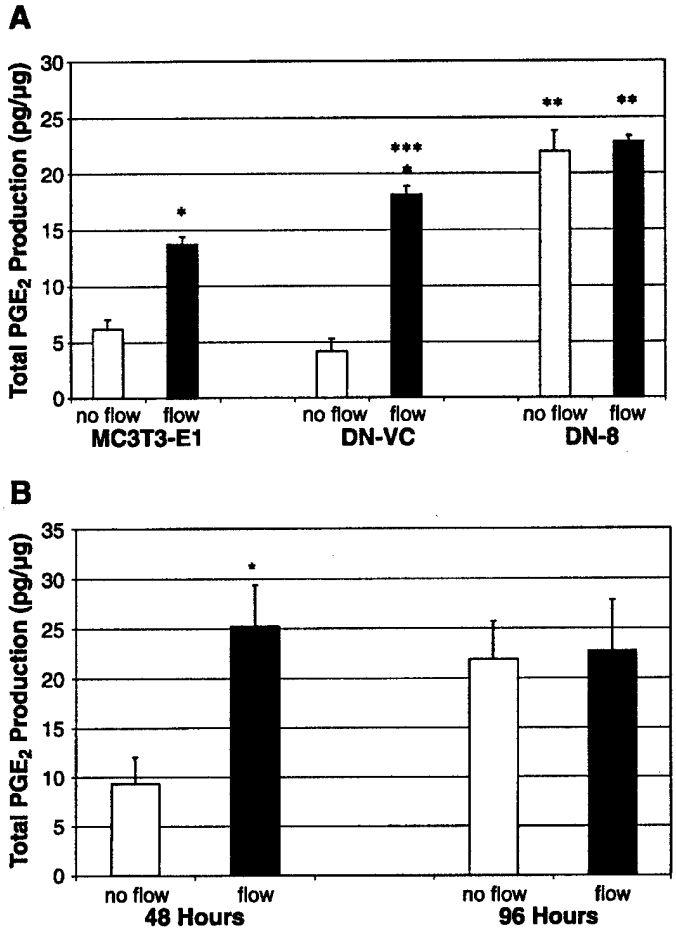


Fig. 4. Results of prostaglandin $E_2$ ($PGE_2$) quantification in the 3 cell lines examined. The numbers are representative of total $PGE_2$ accumulation in the media normalized to total protein and collected after 1-h incubation period. *A*: at 96 h, the MC3T3-E1 and DN-VC cell lines responded to fluid flow with an increase in $PGE_2$ accumulation, whereas the DN-8 cell line did not respond to fluid flow. Although results are shown for the 1-h collections only, similar trends were exhibited in the 0-h collections (data not shown). Interestingly, baseline levels were elevated in this line at this time point. *B*: at 48 h, the DN-8 cell line displayed an increase in $PGE_2$ accumulation in response to oscillatory fluid flow with more accumulation obtained from the 1-h postflow samples. At 96 h, the DN-8 cell line responded to flow with no significant increases in $PGE_2$ accumulation from either the 0-h or 1-h collections. All results shown are plotted as means ± SE with each value representative of at least 10 experiments. *Significantly different from no-flow control within group; **significantly different from media collected 1-h postflow at same time point in DN-VC and MC3T3-E1; ***significantly different from media collected 1-h postflow at same time point in MC3T3-E1.

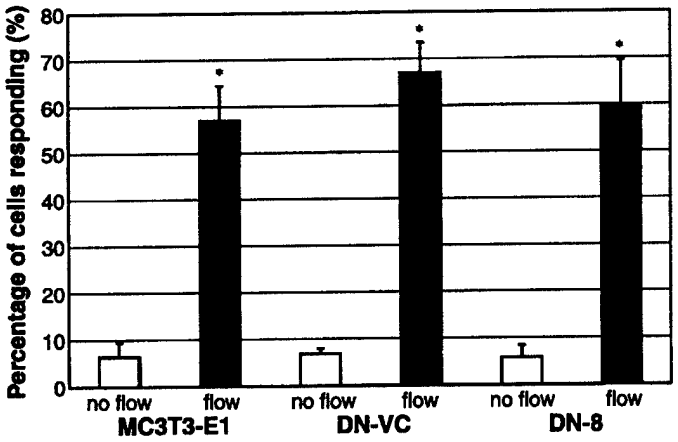


Fig. 5. Results of cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) imaging at 96 h. In all cell lines examined, oscillatory fluid flow induced a significant increase in the percentage of cells responding with an increase in $[Ca^{2+}]_i$ ($P < 0.0003$, at least). Significant differences were not observed between the groups when comparing the no-flow controls or the flowed samples. All results are shown plotted as means ± SE with each value representative of at least 4 experiments (MC3T3-E1) or 6 experiments (DN-VC and DN-8). *Significantly different from no-flow control within group.

role in the mediation of oscillatory fluid flow-induced $PGE_2$ production in osteoblastic cells.

To verify the effectiveness of the dominant-negative strategy used to render the DN-8 cells communication deficient, we quantified the extent of coupling in the DN-8 cells at 24, 48, and 96 h in culture. These results were compared with coupling experiments conducted at the same time points in the communication-intact, control-transfectant DN-VC cell line. We found an 80.1% decrease in coupling in the DN-8 cells between 48 and 96 h in culture, whereas no significant change was noted in the DN-VC cells over the same time period. These results indicate that GJIC in only the DN-8 cell line was dependent on time in culture. Therefore, because the cells are genetically identical and cultured under the same culture conditions, these cells provide a novel model system in the analysis of GJIC in bone cell responsiveness to fluid flow.

To address the role that gap junctions play in the oscillatory fluid flow-induced $PGE_2$ response, we subjected the cell lines to 1 h of oscillatory fluid flow and measured $PGE_2$ accumulation in the media compared with $PGE_2$ accumulation in media from no-flow controls. At the 48-h time point, when intact GJIC was exhibited in the DN-8 cell line, the application of oscillatory fluid flow resulted in significant increases in $PGE_2$ accumulation. However, at the 96-h time point, when GJIC was inhibited, no increase in oscillatory fluid flow-induced $PGE_2$ accumulation resulted. In contrast, the DN-VC cell line responded at both time points with significant increases in $PGE_2$ accumulation. Thus we found that a breakdown in coupling was accompanied by a significant decrease in $PGE_2$ responsiveness to oscillatory fluid flow. These findings strongly suggest that gap junctions and GJIC are necessary in the signal transduction pathway whereby osteoblastic cells increase production of $PGE_2$ in response to oscillatory fluid flow and that a GJIC-dependent pathway exists.

To address the role that gap junctions play in the mediation of oscillatory fluid flow-induced changes in $[Ca^{2+}]_i$, we subjected the cell lines to 3 min of oscillatory fluid flow and measured $[Ca^{2+}]_i$ compared with $[Ca^{2+}]_i$ of no-flow controls. At the 96-h time point, DN-8 and DN-VC cells responded to oscillatory fluid flow with significant increases in $[Ca^{2+}]_i$. Moreover, differences in flow-induced $[Ca^{2+}]_i$ were not significantly different in the DN-8 and DN-VC lines at this time point. Thus we found that a breakdown in coupling was not accompanied by a significant change in $[Ca^{2+}]_i$ and that the $Ca^{2+}$ responses of the cell lines, regardless of degree of coupling, were equally responsive. These findings strongly suggest that gap junctions and GJIC are not necessary in the signal transduction pathway whereby osteoblastic cells increase $[Ca^{2+}]_i$ in response to oscillatory fluid flow and that a GJIC-independent pathway exists.

In this study, we found that although coupling-deficient osteoblastic cells responded to the application of oscillatory fluid flow with significant increases in $PGE_2$ release, changes in $[Ca^{2+}]_i$ were not found due to changes in coupling. These findings suggest that the $PGE_2$ and $[Ca^{2+}]_i$ responses elicited via oscillatory fluid flow may be unlinked in these osteoblastic cells, a notion contradictory to prevailing opinion. To address this issue, we subjected the cell lines to oscillatory fluid flow in the presence of thapsigargin. We found that the introduction of thapsigargin did not significantly affect $PGE_2$ production, whereas the $Ca^{2+}$ response was completely annihilated (data not shown). Furthermore, because we have data indicating that the only source of $Ca^{2+}$ in the MC3T3-E1 cells is from intracellular stores (42) that are emptied by the thapsigargin, our findings provide concrete evidence to suggest a separation of pathways is involved in $Ca^{2+}$ wave propagation and $PGE_2$ production in osteoblastic cells.

In this study, we set out to investigate the role of gap junctions in mediating oscillatory fluid flow-induced

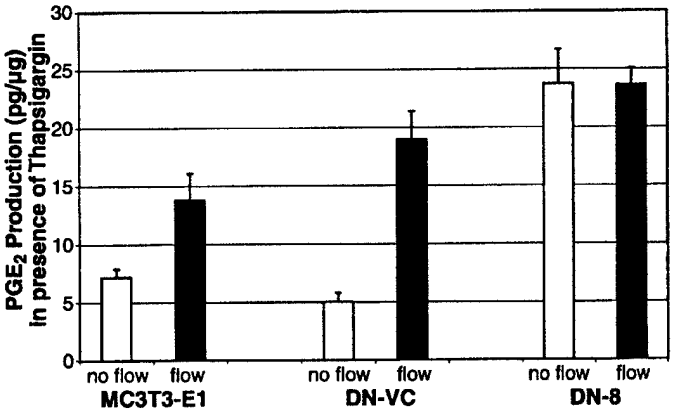


Fig. 6. Results of $PGE_2$ quantification in the presence of thapsigargin at 96 h in the 3 cell lines examined. At 96 h, the $PGE_2$ response of the cell lines to oscillatory fluid flow was not altered by the presence of thapsigargin. All results are shown plotted as means ± SE with each value representative of a minimum of 2 experiments.

$PGE_2$ response in osteoblastic cells. Inasmuch as this was our goal, we were largely interested in whether the application of oscillatory fluid flow resulted in significant increases in $PGE_2$ production in the cell lines. However, studies have shown that the exact $PGE_2$ time course has yet to be elucidated and that flow-induced $PGE_2$ production is not obliterated with the cessation of the stimulant (22). To address the time-dependent response of oscillatory fluid flow-induced $PGE_2$ release in these cell lines, $PGE_2$ accumulation in the media was measured at two time points after cessation of flow. In the first approach, the flowed media were collected immediately after flow exposure; in the second approach, the flowed cells were placed in an equivalent volume of fresh media immediately postflow and incubated for 1 h. We found that in the coupling-intact DN-VC cells, flow-induced levels of $PGE_2$ accumulation in media from cells incubated for 1 h postflow were significantly higher compared with media from cells collected immediately postflow. Similarly, $PGE_2$ levels in media from no-flow control cells incubated for 1 h postflow were significantly elevated compared with levels from no-flow control cells collected immediately postflow. These findings were similar to those observed in the DN-8 cell line at 48 h when coupling was still intact, suggesting that a comparison of baseline $PGE_2$ accumulation levels from media collected from incubated postflow cells is more appropriate than in media collected immediately postflow and may be more sensitive to extracellular regulation.

Curiously, we found that basal $PGE_2$ levels were elevated in the DN-8 cells at the 96-h time point. Although we are unable to definitively explain this result, it is unlikely that the elevation was a result of the transfection process, since the control transfectant DN-VC cells did not exhibit a similar trend. To further address this issue, we added the ionophore 4-bromocalcium (50 $\mu$M for 15 min) to confluent slides of DN-8 cells and measured $PGE_2$ accumulation levels in excess of those shown in Fig. 4 (data not shown), indicating that increases beyond these basal levels were indeed possible. In any case, we do not feel that these findings detract from the main finding of this paper, namely that GJIC-deficient cells do not respond to oscillatory fluid flow with an increase in $PGE_2$ release.

It is also possible that factors other than GJIC are involved in the responsiveness of a cell ensemble to oscillatory flow by affecting the inherent responsiveness of the individual cells. For instance, it is possible that membrane permeability or morphological changes in the membrane are important and could lead to sensitivity changes in protein receptors, ion channels, and cytoskeletal elements. This may help to explain the increase we observed in basal $PGE_2$ production levels in the DN-8 cells at the 96-h time point. Furthermore, it is also possible that GJIC may affect such changes in cellular sensitivity. For instance, it has previously been shown that GJIC contributes indirectly to morphological changes by contributing to extracellular matrix organization (3).

Although a substantial body of evidence exists linking GJIC and cellular responsiveness to physical stimuli, the work to date has provided only indirect evidence. For instance, several studies have shown that the application of physical stimuli in vitro results in increased Cx43 expression in both osteoblasts (43) and osteocytes (29), a finding that parallels those in smooth muscle (5) and endothelial cells (11). However, these studies do not address changes in coupling or that changes in coupling influences the sensitivity of the cell ensemble. Thus it is important to distinguish between connexin formation and functional coupling, which would provide direct evidence to suggest that gap junctions are important in mechanotransduction.

In this study, we investigated the role of GJIC in oscillatory fluid flow-induced $PGE_2$ production and changes in $Ca_i^{2+}$ signaling. We found direct evidence to indicate that the $PGE_2$ response was dependent on gap junctions, demonstrated by the lack of $PGE_2$ released in the gap junction-deficient DN-8 cell line compared with the DN-VC cell line. In addition, by investigating real-time $Ca_i^{2+}$ responses in these cell lines, we found that all three cell lines were able to respond to oscillatory fluid flow with an immediate increase in $[Ca^{2+}]_i$. Finally, by blocking the $Ca_i^{2+}$ response with thapsigargin, we demonstrated that the $PGE_2$ response in MC3T3-E1 cells to oscillatory fluid flow does not depend on an increase in $[Ca^{2+}]_i$. Together, these findings strongly suggest an important role for gap junctions and GJIC in bone cell mechanotransduction mechanisms.

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# FLUID FLOW-INDUCED PROSTAGLANDIN $E_2$ RESPONSE OF OSTEOBLASTIC ROS 17/2.8 CELLS IS GAP JUNCTION-MEDIATED AND INDEPENDENT OF CYTOSOLIC CALCIUM

(Bone in press)

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**Running Heading:** Flow-induced $PGE_2$ and $Ca^{2+}$ response in ROS cells

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**Figures:** 1 Table
6 Figures

## ABSTRACT

Mechanotransduction is the process by which a bone cell senses a biophysical signal and elicits a response. While it has been demonstrated that bone cells can respond to a wide variety of biophysical signals including fluid flow, stretch and magnetic fields, the exact pathways and mechanisms involved are not clearly understood. We postulated that gap junctions may play an important role in bone cell responsiveness. Gap junctions (GJ) are membrane spanning channels that physically link cells and support the transport of small molecules and ions in the process of gap junctional intercellular communication (GJIC). In this study we exmained the role of GJ and GJIC in mechanically stimulated osteoblastic cells. Using steady and oscillatory fluid flow, mechanical stimulation was applied to ROS 17/2.8 cells and a derivative of these cells expressing antisense cDNA for the gap junction protein connexin 43 (RCx16) which possess significantly different levels of GJIC and quantified $PGE_2$ and $Ca^{2+}$ responses. We found that osteoblastic cells possessing increased GJIC also exhibited increased $PGE_2$ accumulation in supernatant following fluid flow stimulation. Interestingly, we found that neither osteoblastic cell line responded to the fluid flow with an increase in cytosolic calcium. Thus, our results suggest that GJ and GJIC may be important in the mechanotransduction mechanisms by which $PGE_2$ is mechanically induced in osteoblastic cells independent of cytosolic calcium.

# INTRODUCTION

In fully developed bone tissue, osteoblasts are important in maintaining the formation of new bone in the continual process of bone turnover, while osteoclasts are important in the removal of existing bone. At the cellular level, researchers have proposed that in order to accomplish this turnover, the bone cells must be sensitive to loading effects. In this model, load induced from the global environment reaches the cells at the local level in the form of a biophysical signal which can affect the cell to bring about a response (mechanotransduction). How these processes are mediated remains to be determined.

Gap junctions (GJ) are formed from the docking of protein channels (connexons) in neighboring cell membranes. This physical linking enables cell-to-cell exchange via the rapid diffusion of small molecules and ions (<1kDa) in a process known as gap junctional intercellular communication (GJIC). With the exception of blood cells and muscle cells, gap junctions are abundant in most cell types including bone-forming osteoblasts. Although gap junctions have been linked to developmental and morphogenic processes, their role in developed tissues remains unclear (Vander Molen). To this end, researchers have recently begun to investigate the regulatory role of GJ and GJIC in such fully developed tissues as bone and it has been determined that, in the immortalized rat osteoblastic sarcoma, ROS 17/2.8 (ROS) cell line, GJIC is pivotal in proliferation and differentiation (Donahue), as well as, hormonal responsiveness (Vander Molen) and gene expression (Civitelli). Furthermore, GJIC contributes to human fetal osteoblastic cell differentiation (Donahue, 2000).

In bone, osteoblasts are connected to other osteoblasts and osteocytes via GJ. Although the rationale for this is not clearly understood, it is possible that the GJ mediate the cellular response by diffusing a signal throughout the bone tissue. This in turn enables networks of bone

cells to react in syncytium to a physical stimuli thereby amplifying and/or orchestrating the response. In support of this concept, Ziambaras, et al. found that GJIC between ROS cells was enhanced by cyclic stretch (Ziambaras). Furthermore, we have found that while osteoblastic cells respond to physiologic levels of fluid flow with increases in cytosolic calcium concentration ($[Ca^{2+}]_i$), cyclic stretch levels must be far in excess of the physiologic realm to elicit a response (You), suggesting that fluid flow may be the more appropriate biophysical stimulus. We have recently shown in MC3T3-E1 cells that oscillatory fluid flow-induced prostaglandin $E_2$ ($PGE_2$) production, indicative of bone mineralization, correlates with GJIC (Saunders). To our knowledge this is the first direct evidence demonstrating a link between mechanotransduction and GJIC.

The purpose of the current study was two-fold. First, we set out to investigate the role of GJ and GJIC in oscillatory fluid flow (OFF)-induced responsiveness in two ROS cell lines with significantly different levels of communication (GJIC), which we established using fluorescent dye transfers and flow cytometry. We quantified OFF-induced changes in $PGE_2$ and $[Ca^{2+}]_i$, markers selected for their role in bone turnover (2 refs). These findings, in conjunction with our previous work allowed us to draw more general conclusions about the role of GJ and GJIC in osteoblastic responsiveness rather than cell line specific inferences.

Second, we set out to determine if the ROS-derived lines were capable of eliciting a $Ca^{2+}$ response induced via either steady flow (SF) or OFF at various frequencies. Although previous findings suggest that ROS cells do not respond to SF with increases in $Ca^{2+}$, these studies were completed in a very limited number of cells and therefore were not conclusive (Allen). Furthermore, the effect of the more physiologically appropriate OFF response has not been addressed. In conjunction with this aim, we wanted to determine if the OFF-induced $PGE_2$

response in the ROS-derived lines was calcium dependent. Our findings in the MC3T3-E1 cells suggest that the $PGE_2$ pathway does not involve calcium, a curious and novel finding which we wanted to further examine.

## MATERIALS AND METHODS

*Cell Culture*

The immortalized osteoblastic cell lines, ROS 17/2.8 and RCx16 were utilized in this study. ROS 17/2.8 cells (ROS) are rat osteoblastic sarcoma cells expressing phenotypic characteristics of fully differentiated osteoblasts ( ) whereas RCx16 cells are ROS cells rendered communication-deficient by the stable transfection with antisense cDNA to connexin 43 (Cx43), the predominant gap junction protein in bone (Van der Molen). ROS cells were cultured in Ham's F12 (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 1% penicillin/streptomycin (P/S) (GIBCO BRL, Grand Island, NY), 1% sodium pyruvate and 1% glutamine. RCx16 cells were cultured in ROS media supplemented with geneticin (G418 400μg/mL) to maintain antibiotic selection of antisense-expressing cells. Cells were maintained in an incubator at 37°C and 5% $CO_2$ with steady and oscillatory flow experiments conducted in the appropriate media supplemented with 2% FBS using ROS and Rcx16 cells with passage numbers less than 20 for all experiments.

*Cell Preparation*

Cell preparation was conducted as previously described for osteoblastic MC3T3-E1 cells (Saunders). Briefly, for $Ca^{2+}$ experiments, cells were plated on quartz slides ($1.0x10^5$ cells/slide) and allowed to reach 75-80% confluence over 24-48 hours; for $PGE_2$ experiments, cells were

plated on microscope slides ($3.5x10^5$ cells/slide) and allowed to reach 85-90% confluence over 24-48 hours. GJIC experiments were prepared as described for the $PGE_2$ experiments and cells for fluorescent microscopy and flow cytometry were cultured concurrently.

*Experimental Methods*

*Gap Junctional Intercellular Communication Coupling Assays*

Functional GJIC assays were qualified using a double labeling fluorescent dye technique as previously described (Donahue) (Yellowley) and quantified using flow cytometry. Donor cells labeled with a fluorescent dye mixture containing 20μL of calcein AM (Molecular Probes, Eugene, Oregon) and 7μL of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes, Eugene, Oregon) in 2mL of bovine serum albumin- (BSA) enriched PBS with 20μL of pluronic acid (Molecular Probes, Eugene, Oregon) were dropped onto glass slides containing confluent monolayers of unlabeled cells at a ratio of approximately 1:500 cells (labeled to unlabeled) and incubated for 90 minutes at 37°C. Cells were fluorescently visualized using a Nikon fluorescent microscope (Nikon EFD-3, Optical Apparatus Co., Ardmore, PA) with fluorescein ($\lambda_{excitation}$ = 465-495nm; $\lambda_{emission}$ = 520nm) and rhodamine ($\lambda_{excitation}$ = 541-551nm; $\lambda_{emission}$ = 590nm) filters to locate the calcein and DiI loaded cells, respectively. In the dual labeling technique, the extent of coupling (communication) was determined by counting the number of neighboring cells fluorescing green from a single donor cell, while the DiI was used to distinguish the labeled cells from those in the monolayer. Ten cells were randomly selected and counted for each coverslip and a maximum of fifteen cell transfers for each cell were counted.

Inasmuch as the dual labeling technique was semi-quantitative, flow cytometry was employed to quantify larger cell volumes. Flow cytometry was employed following the dual labeling technique with minor modifications. For cytometric experiments, donor cells were dropped onto cells in monolayer at a ratio of 1:4 cells (labeled to unlabeled). Following the 90 minute incubation period, cells were trypsinized, centrifuged and resuspended in media supplemented with 2% FBS at a density of $1x10^6$ cells/mL. Cytometry was accomplished with a single laser (488nm) benchtop flow cytometer (Coulter XL). Samples were run at 300 cells/sec with each sample consisting of approximately 50,000 cells. Prior to cytometric analysis, controls were first run to establish a baseline for gate parameter selection. That is, for both cell lines, the following controls were run: no dye; calcein only; DiI only; and, calcein and DiI. Once run, the cell fluorescences were grouped such that definitive boundaries were set between the cells in each of these groups (Figure 1). With the gates in place, the double labeled samples were run through the cytometer and the percentage of individual cells falling within each of the regions were quantified.

*Parallel Plate Flow Chambers and Testing Machine*

For $PGE_2$ experiments, bone cells were placed in a parallel plate flow chamber (Frangos, Hung, Jacobs, You) and subjected to oscillatory fluid flow. For $Ca^{2+}$ experiments, cells in the chamber were subjected to either steady or oscillatory fluid flow resulting in a shear stress of $20dyne/cm^2$. During the OFF exposure, the chambers were connected to a pneumatic, closed-loop feedback materials testing machine (EnduraTec, Minnetonka, MN) via tubing and syringes with OFF delivered via a 1 or 2Hz sine wave. For $Ca^{2+}$ SF experiments, the chambers were connected to a Harvard syringe pump (Harvard Apparatus, South Natick, MA). Flow probes

(Transonic Systems Inc., Ithaca, NY) were used to monitor flow rate and shear stress was determined from the equation for parallel plate flow as previously described (Frangos, Saunders) being a function of flow rate, fluid viscosity and chamber dimensions.

*$PGE_2$ Quantification*

Cell $PGE_2$ release in media collected from the chamber ports was quantified with a commercially available, competitive binding enzyme immunoassay system (BioTrak, Amersham Pharmaceuticals, Piscataway, NJ). Cells were exposed to 1 hour of oscillatory flow at either 1 or 2 Hz. Following flow, approximately 1mL of media was collected from the inlet and outlet ports of the parallel plate flow chamber and frozen at –80°C for a period not exceeding 1month. In addition, the ionophore, 4-Bromo-Calcium (50uM), was added to a plated slide from each cell line for 15 minutes and incubated at 37°C. The media from these collections served as positive controls in the $PGE_2$ assays and were repeated for each assay.

For comparison purposes, $PGE_2$ accumulation in the supernatant was normalized to total protein (BioRad, Hercules, CA). Following collection of the media, cells were removed from the microscope slides by trypsinization, centrifuged at 200G for 8 minutes, resuspended in 0.5mL of 0.05% Triton X 100 detergent and lysed using three cycles of rapid freezing (-80°C) and thawing. Optical densities of the samples were read at 405nm using a microplate reader. Manufacturer-supplied standards were analyzed and used to construct the standard curve from which the sample concentrations were read.

*Calcium Imaging*

$Ca^{2+}$ imaging was completed with epifluorescent microscopy and the dual-wavelenth ratiometric dye, fura2-AM (Molecular Probes, Eugene, OR) as previously described (Saunders) (You)(Jacobs). Briefly, preconfluent slides of cells were loaded with 10uL (10uM) fura2-AM in 1mL of fresh media supplemented with 2%FBS and incubated at 37°C for 45 minutes. Following incubation, cells were washed, inverted on the parallel plate flow chamber and transferred to a fluorescent microscope where they were connected to the flow delivery system. Cells were subjected to 3min of steady or oscillatory flow preceded by a 1min no flow baseline. An image acquisition and analysis software package (Metafluor, Universal Imaging Corporation, West Chester, PA) was used to capture the images and convert the fluorescent intensities at the two wavelengths into ratios of bound to unbound $Ca^{2+}$, and from there, real time $[Ca^{2+}]_i$.

*Data Analysis*

$Ca^{2+}$ results were analyzed with a Rainflow counting technique (Jacobs) as previously described with a threshold response defined as a $[Ca^{2+}]_i$ of 20nM or larger. $PGE_2$ results were analyzed using a microplate reader and normalized to total protein with total production presented in pg/μg. GJIC from dual labeling was quantified by counting cell fluorescence transfers, as previously described and flow cytometry was quantified by determining the percentage of cells communicating. All $Ca^{2+}$, $PGE_2$ and GJIC data obtained were expressed as mean ± SEM. To compare results between the cell lines, GLM ANOVAs with SNK post-hoc comparisons were completed using a commercially available software program (Instat, GraphPad Software Inc., San Diego, CA) with an *a priori* significance level of 0.05.

## RESULTS

*GJIC Quantification*

GJIC was qualitatively and quantitatively evaluated in the ROS and RCx16 cell lines. Cell transfers from the double labeling assays were quantified for the cell lines (Figure 2). Shown plotted is the mean±SEM number of individual acceptor cells in monolayer fluorescing green from an individual donor cell. For any given donor cell, a maximum of fifteen transfers were counted and a minimum of 360 donor cells from each line were examined in four independent experiments. We found that the ROS cells were significantly more coupled than the RCx16 cells exhibiting a 3.8-fold increase in number of cell transfers. Cytometric results (Figure 3) were in agreement with the microscopy experiments and showed that the ROS cell line was significantly more coupled than the RCx16 cell line ($p<0.0003$) demonstrating that approximately 20% of the ROS cells were capable of communicating compared to 10% of RCx16 cells.

*$PGE_2$ Response to Oscillatory Fluid Flow*

Media from ROS and RCx16 cells collected immediately following flow accumulated significantly more $PGE_2$ than cells not exposed to flow (Figure 4). Fluid flow at 1Hz induced a 6.6-fold increase in $PGE_2$ accumulation in ROS cells in comparison to its no flow controls and induced a 3.4-fold increase in $PGE_2$ accumulation in RCx16 cells in comparison to its no-flow controls. This finding was frequency dependent with media from ROS and RCx16 cells collected immediately following flow at 2Hz accumulating significantly more $PGE_2$ than their counterparts flowed at 1Hz (1.5-fold and 2.4-fold increases, respectively). In addition, for both

frequencies, media from coupling-intact ROS cells collected immediately following flow accumulated significantly more $PGE_2$ than media from the coupling-deficient RCx16 cells. ROS cells exhibited for the three cases, a 1.6-fold increase, a 1.2-fold increase and a 1.9-fold increase in $PGE_2$ accumulation in comparison to the RCx16 cells from the no-flow controls, OFF at 1Hz and OFF at 2Hz, respectively.

*Calcium Response to Steady and Oscillatory Fluid Flow*

An oscillatory fluid flow-induced calcium response was not exhibited in either the ROS or the RCx16 cells regardless of waveform and frequency. Table 1 lists the type of flow regime (SF = steady flow; OFF = oscillatory fluid flow), frequency and number (n) of experiments conducted for each of the two cell lines. In all cases, a calcium response was not induced via the fluid stimulation and each experiment contained a minimum of 45 cells in the field of view. Additional studies were conducted (data not shown) using FBS at 5% and 10%, as well as Tyrodes flow media. In our hands, under none of these conditions outlined were we able to induce a calcium response to fluid flow stimulation.

To determine if this lack of a response was inherent in the cell line or specific to fluid flow, we added the calcium ionophore, 4-Bromo-Calcium, directly onto the slide of cells resting on the microscope stand and measured changes in calcium as a result of the ionophore. As shown in Figure 5, the cells responded to the ionophore with a robust increase in $Ca^{2+}$. This result was typical of both the ROS and RCx16 cell lines and strongly suggests that these ROS-derived cell lines lack a flow-induced (SF and OFF) $Ca^{2+}$ response.

## DISCUSSION

We undertook this study with two aims. First, we wanted to examine the role of GJ and GJIC in oscillatory fluid flow (OFF)-induced responsiveness in two ROS cell lines with significantly different levels of GJIC. Second, we set out to determine if the ROS-derived lines were capable of eliciting a $Ca^{2+}$ response induced via various waveforms and frequencies of fluid flow and whether the induced $PGE_2$ response in the ROS-derived lines was calcium dependent.

With respect to the first aim, we found that osteoblastic cells with intact GJIC (ROS) responded to oscillatory fluid flow with a robust increase in $PGE_2$ accumulation as measured in the flow medium. Osteoblastic cells with disrupted GJIC (RCx16) responded to oscillatory fluid flow with a less responsive increase in $PGE_2$ accumulation as measured in the flow medium. These responses were frequency dependent and significantly increased at 2Hz in comparison to 1Hz. We recently found similar results in the mouse osteoblastic MC3T3-E1 cell line expressing a dominant/negative Cx43 (Saunders). Taken together, these findings strongly suggest that GJ and GJIC play a role in mechanotransduction whereby bone cells respond to mechanical stimuli with an increase in $PGE_2$ and that this response in osteoblasts may be independent of cell line. Moreover, this work adds to a growing body of direct evidence indicating that gap junctions link cellular responsiveness to extracellular stimulation, both physical and chemical (Van der Molen)(McLeod).

It is interesting to note that the response of the ROS cell lines to fluid flow was quite robust. In previous research, we and others have added a 1hr post-incubation period following the removal of the flow stimulus. We have found that whereas the immediate measurement of $PGE_2$ in most osteoblastic cells is very modest, the incubated response is quite significant. In the ROS lines, the additional incubation period was not required. This may suggest that the timeline

for $PGE_2$ accumulation is cell line dependent or that cells with an attenuated calcium response in the presence of fluid flow compensate with an amplified $PGE_2$ response. Although we acknowledge that a second messenger is required to elicit the $PGE_2$ response, it may be that some other signaling molecule, such as cAMP, may be involved. It is important to delineate that the calcium measured in the current study was measured over a 3min duration. Our findings do not omit the possibility that calcium is involved in further downstream regulation following the initial calcium-independent response.

With respect to the second aim, we found that neither the ROS nor RCx16 cell lines were capable of responding to fluid shear with an increase in cytosolic calcium. Steady and oscillatory waveforms were applied and frequencies of 1 and 2Hz were utilized. These results suggest that the ROS cell lines inherently lack a fluid flow-induced calcium response. As a corollary, these findings suggest that the calcium response in ROS and RCx16 cells are not GJ and GJIC dependent. Interestingly, this is also in agreement with our previous work whereby MC3T3-E1 cells displaying varying degrees of GJIC were capable of responding to fluid flow with similar increases in cytosolic calcium. These findings have immediate import in that the ROS cell line is a novel system for dissecting mechanotransduction pathways in bone cells. By inherently lacking a calcium response, these cells annihilate the effects of flow-induced calcium mobilization without requiring nonspecific pharmacological agents.

Interestingly, we found that the OFF-induced $PGE_2$ response in the ROS-derived lines was independent of changes in cytosolic calcium. With our previous work in MC3T3-E1 osteoblastic cells, the same conclusion was reached. This has important implications in that this phenomenon may not be merely cell line dependent but may hold true for osteoblastic lines in general. The question then arises as to how $PGE_2$ may be increased independent of changes in

$[Ca^{2+}]_i$ in ROS cells. Indeed, previous studies have shown that ROS cells have a robust cytosolic $Ca^{2+}$ response to several extracellular signals including parathyroid hormone, vitamin D, ATP and bradykinin (add refs here Donahue, 1986), suggesting that they have the machinery to respond to extracellular signals, other than fluid flow, with an increase in $[Ca^{2+}]_i$. Recently purinergic P2 receptors have been identified on bone cells including osteoblasts and osteoclasts. Although their function is not completely understood, they may act with ATP in a local messenger system (Hoebertz). Jorgensen, et al found that human osteoblasts required P2 receptors in the propagation of fast calcium waves and once blocked, slow wave propagation was gap junction dependent. Our results support their findings, whereby the $PGE_2$ produced was gap junction dependent, and taken with our previous work, suggest that not only is this response independent of calcium, but the calcium response may be either intact (MC3T3-E1) or completely absent (ROS17/2.8 and RCx16).

## CONCLUSIONS

In this study we exposed two osteoblastic cell lines to steady and oscillatory fluid flow to determine the role of gap junctions in mediating mechanical stimulation. We found that while the $PGE_2$ response was contingent upon the presence of functioning gap junctions, the $[Ca^{2+}]_i$ response was not. Furthermore, we found that the osteoblastic cell lines examined in this work were incapable of producing a flow-induced $Ca^{2+}$ response regardless of fluid flow media or flow regime (steady and oscillatory). These results suggest that while gap junctions may be important in the mechanotransduction mechanisms whereby $PGE_2$ production is increased, calcium changes in these osteoblastic cell lines were not tightly linked to gap junctions.

## ACKNOWLEDGMENTS

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## LEGENDS

Table 1. Result of calcium imaging. A variety of steady flow (SF) and oscillatory fluid flow (OFF) waveforms were investigated. Shown are the results of flow experiments conducted in 2% FBS. Neither SF nor OFF at 1 or 2Hz elicited a calcium response. Similar findings were obtained for flow experiments conducted in Tyrodes and 5% and 10% FBS (results not shown).

***Figure 1. Schematic of oscillatory fluid flow delivery system. a) Oscillatory fluid flow was delivered via a sinusoidal waveform generated by a materials testing machine connected to the flow chamber using tubing and syringes. b) The flow chamber consisted of a parallel plate design. Cells in monolayer on the glass slide were inverted on the flow chamber on a silastic gasket. During fluid flow, the assembly was held together with either a vacuum seal or encased in a polycarbonate case (not shown).***

Figure 2. Flow cytometric analysis. Using flow cytometry and controls loaded in all possible combinations of dye for both of the cell lines, gates were determined. As shown in the figure, the region was broke into four quadrants. Calcein only cells fluoresced in quadrant 2, while DiI cells fluoresced in quadrant 3. None of the groups of control cells fell within quadrant 4 and thus, coupling was determined to take place within this quadrant. That is, cells increasing fluorescence of calcein, those receiving the dye via gap junctions, shifted up and over from the third quadrant to the fourth. Although it appears that some level of DiI is observed in these cells, we believe it is an artifact of the interaction of the dyes.

Figure 3. Results of double labeling assay in the ROS and RCx16 cell lines. The ROS cells were significantly more coupled than the RCx16 cells ($p<0.035$). Each bar is representative of a

minimum of 180 cells (maximum 240) and is plotted as mean ± SEM with individual cell transfers not counted past a maximum of fifteen cells.

Figure 4. Results of flow cytometry in the ROS and RCx16 cell lines. The ROS cells were significantly more coupled that the RCx16 cells ($p<0.0003$). Each bar is representative of 300,000 cells and is plotted as mean ± SEM and is representative of six experiments.

Figure 5. Results of $PGE_2$ quantification in the cell lines examined. The numbers are representative of total $PGE_2$ accumulation in the media normalized to total protein. Both ROS and RCx16 cells responded to fluid flow with a significant increase in $PGE_2$ production in comparison to the respective no-flow controls. This response was frequency dependent in that cells responded to a 2Hz sinusoidal waveform with a significant increase in $PGE_2$ in comparison to both the no flow controls and the $PGE_2$ production induced at 1Hz. Moreover, in all flow profiles, the ROS cells responded with significantly more $PGE_2$ production that the RCx16 for a given profile. All results are shown plotted as mean ± SEM with each value representative of at least twelve experiments. * - significantly different from no-flow control within group; ** - significantly different from no-flow control and 1Hz waveform

Figure 6. The ionophore 4-Bromo-Calcium induced a large calcium response in the cells indicating that the lack of a calcium response exhibited under fluid flow was inherent in the cell line and not a function of technique.

| | SF | OFF 1Hz | OFF 2Hz |
|---|---|---|---|
| ROS | n=8 | n=12 | n=10 |
| RCx16 | n=4 | n=5 | n=5 |

Table 1 (Saunders, M., et al.)

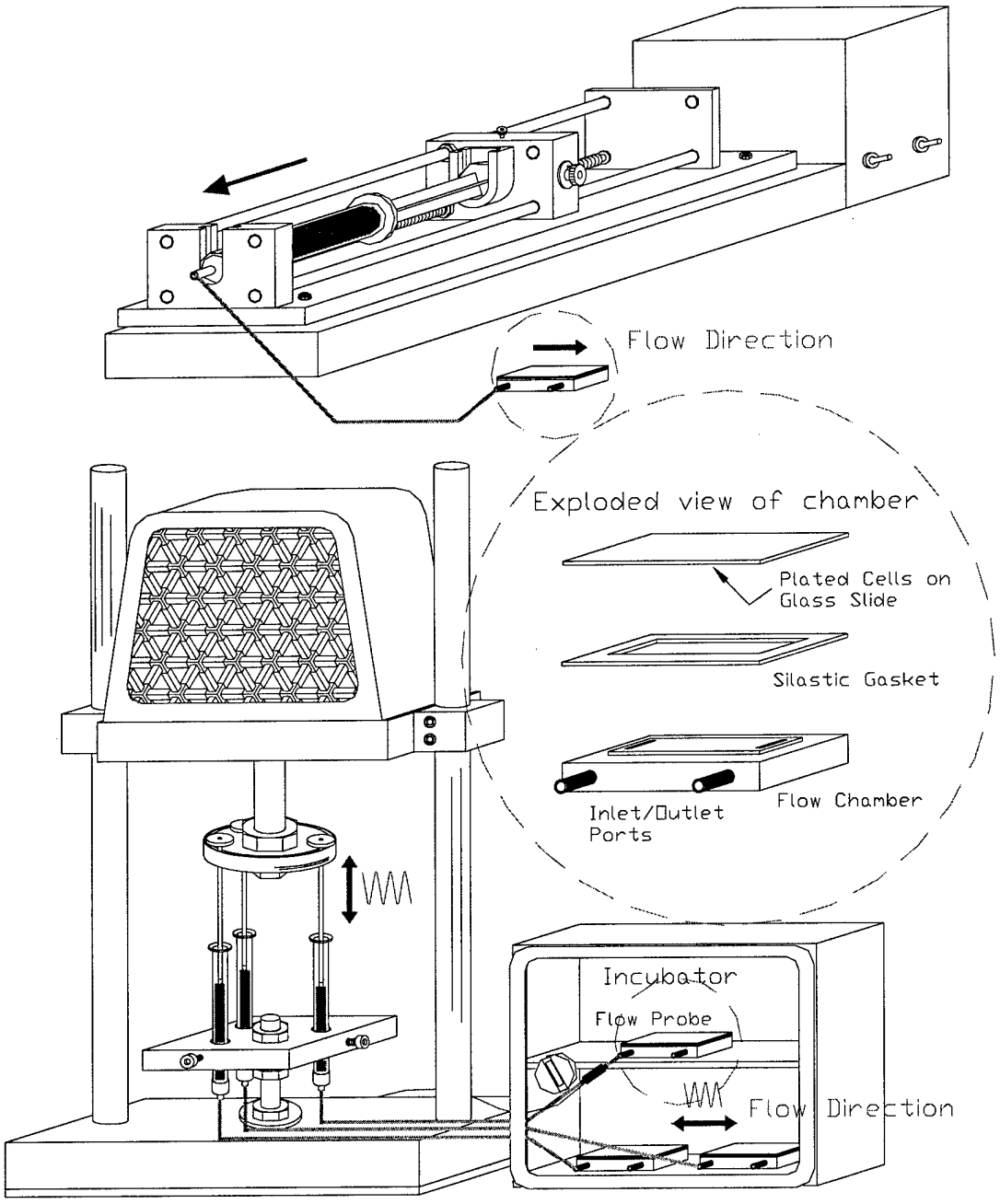


Figure 1 (Saunders, M., et al.)

Cytometry pics

Figure 2 (Saunders, M., et al.)

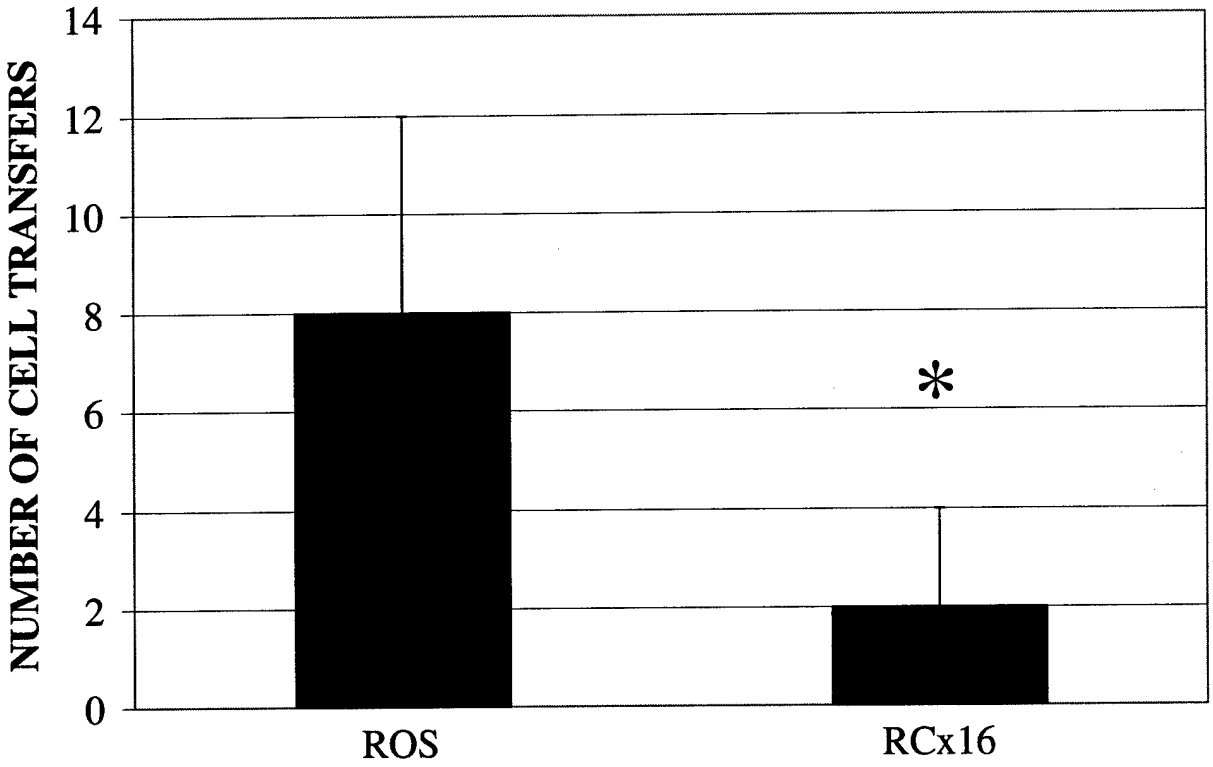


Figure 3 (Saunders, M., et al.)

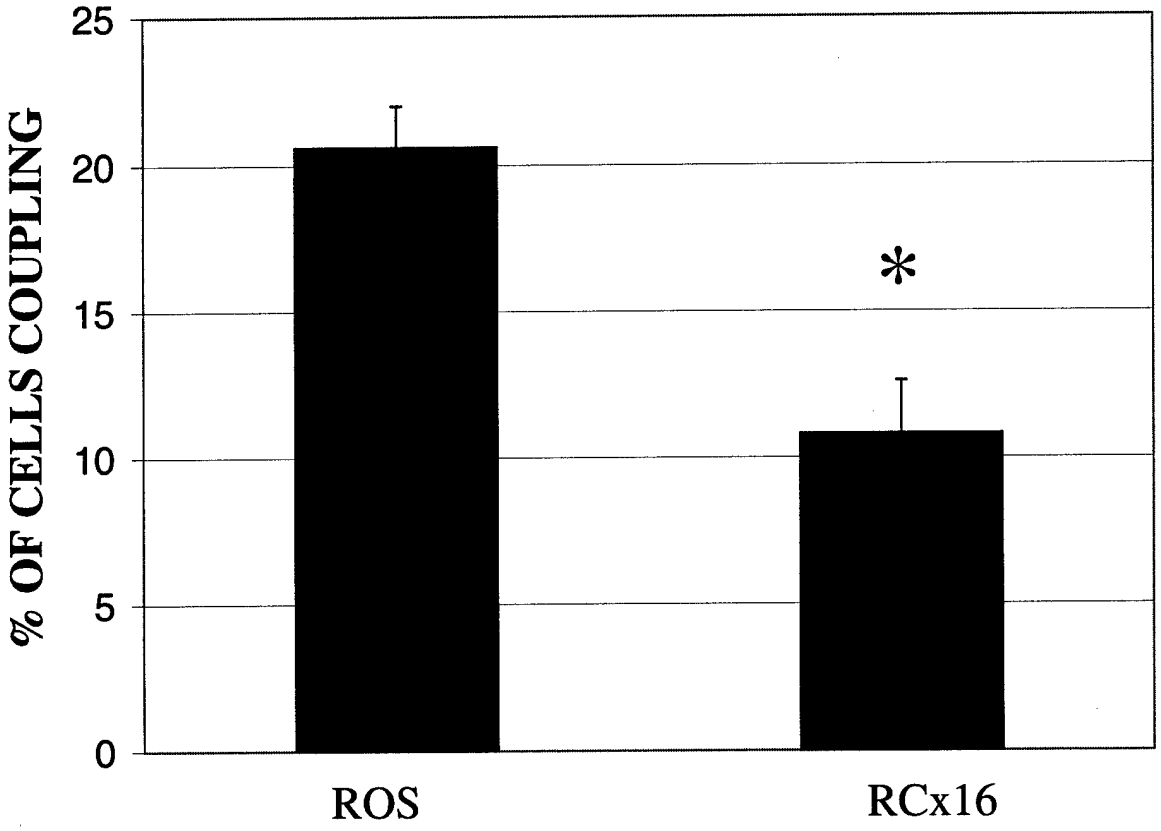


Figure 4 (Saunders, M., et al.)

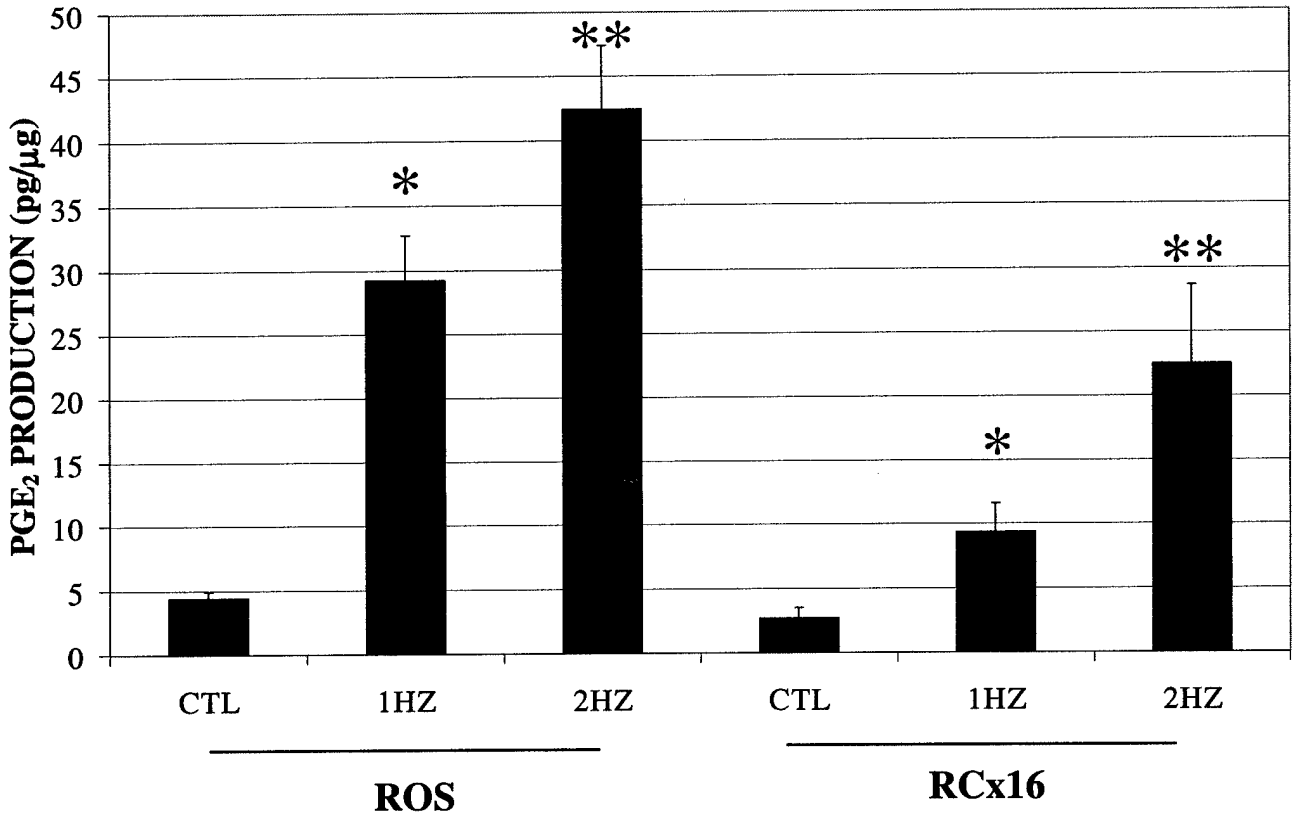


Figure 5 (Saunders, M., et al.)

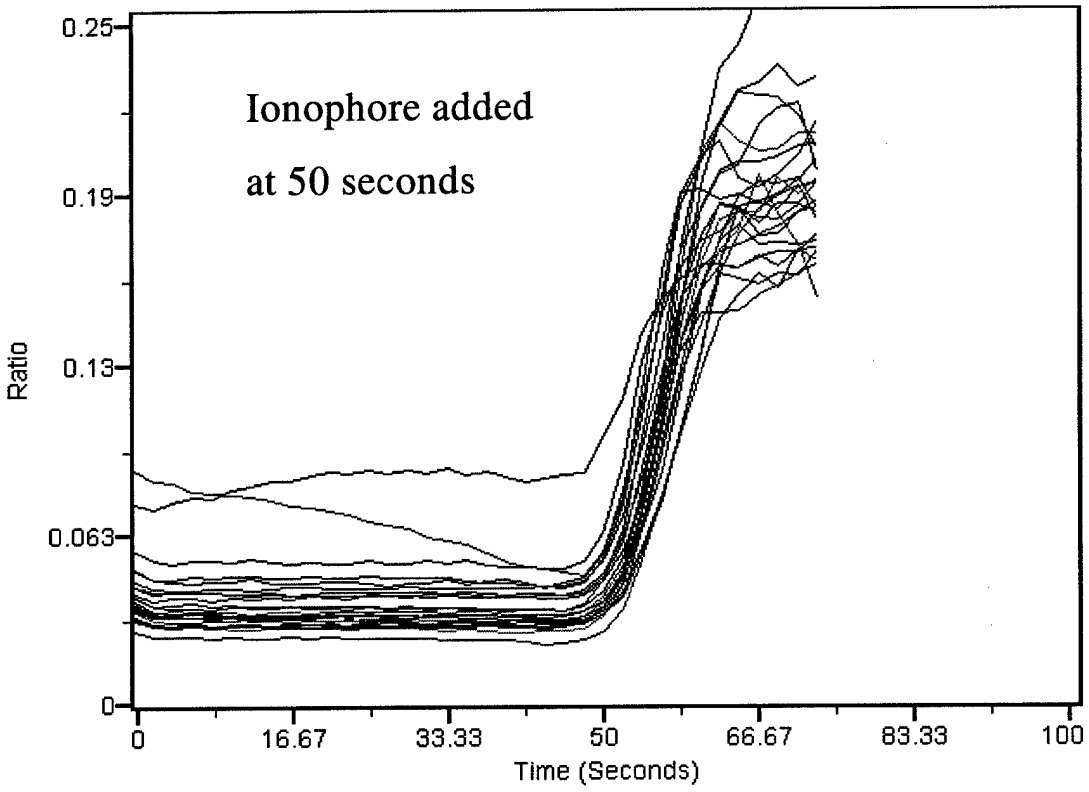


Figure 6 (Saunders, M., et al.)

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# P2Y Purinoceptors Are Responsible for Oscillatory Fluid Flow-induced Intracellular Calcium Mobilization in Osteoblastic Cells*

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**We previously found that oscillatory fluid flow activated MC3T3-E1 osteoblastic cell $Ca^{2+}_i$ mobilization via the inositol 1,4,5-trisphosphate pathway in the presence of 2% fetal bovine serum (FBS). However, the molecular mechanism of fluid flow-induced $Ca^{2+}_i$ mobilization is unknown. In this study, we first demonstrated that oscillatory fluid flow in the absence of FBS failed to increase $[Ca^{2+}]_i$ in MC3T3-E1 cells. Apyrase (10 units/ml), which rapidly hydrolyzes 5′ nucleotide triphosphates to monosphophates, prevented the fluid flow induced increases in $[Ca^{2+}]_i$ in the presence of FBS. Adding ATP or UTP to flow medium without FBS restored the ability of fluid flow to increase $[Ca^{2+}]_i$, suggesting that ATP or UTP may mediate the effect of fluid flow on $[Ca^{2+}]_i$. Furthermore, adenosine, ADP, UDP, or adenosine 5′-*O*-(3-thiotriphosphate) did not induce $Ca^{2+}_i$ mobilization under oscillatory fluid flow without FBS. Pyridoxal phosphate 6-azophenyl-2,4′-disulfonic acid, an antagonist of P2X purinoceptors, did not alter the effect of fluid flow on the $Ca^{2+}_i$ response, whereas pertussis toxin, a $G_{i/o}$-protein inhibitor, inhibited fluid flow-induced increases in $[Ca^{2+}]_i$ in the presence of 2% FBS. Thus, by the process of elimination, our data suggest that P2Y purinoceptors (P2Y2 or P2Y4) are involved in the $Ca^{2+}_i$ response to fluid flow. Finally, a decreased percentage of MC3T3-E1 osteoblastic cells treated with P2Y2 antisense oligodeoxynucleotides responded to fluid flow with an increase in $[Ca^{2+}]_i$, and an increased percentage of ROS 17/2.8 cells, which do not normally express P2Y2 purinoceptors, transfected with P2Y2 purinoceptors responded to fluid flow in the presence of 2% FBS, confirming that P2Y2 purinoceptors are responsible for oscillatory fluid flow-induced $Ca^{2+}_i$ mobilization. Our findings shed new light of the molecular mechanisms responsible for oscillatory fluid flow-induced $Ca^{2+}_i$ mobilization in osteoblastic cells.**

It is well known that mechanical loading of bone results in a variety of biophysical signals that may affect bone cellular metabolism and differentiation (1–3). One of these biophysical signals, extracellular fluid flow, has been demonstrated to be a potent stimulator of osteoblastic cell metabolic activity, differentiation, extracellular matrix protein production, and gene expression (4–8). Recently we reported that oscillatory fluid flow in the presence of 2% fetal bovine serum (FBS),[1] activated MC3T3-E1 osteoblastic cell intracellular calcium ($Ca^{2+}_i$) mobilization via the inositol 1,4,5-trisphosphate pathway and increased steady state levels of osteopontin, a bone extracellular matrix protein, mRNA in a $Ca^{2+}_i$-dependent manner (9). We and others (10) also found that extracellular signaling molecules present in FBS are necessary for fluid flow-induced increases in intracellular calcium ion concentration ($[Ca^{2+}]_i$) in bone cells. However, the nature of these signaling molecules, their receptors, and their roles in oscillatory fluid flow-induced $Ca^{2+}_i$ mobilization are still unclear.

$Ca^{2+}_i$ is an early response second messenger that plays an important role in a number of intracellular signaling pathways and is typically observed to increase dramatically within seconds of stimulation. As a second messenger, $Ca^{2+}_i$ transduces extracellular changes (*i.e.* first messenger) to the cell interior and potentially to the genome and is important in the regulation of a variety of cellular functions (11). For instance, we previously demonstrated that oscillatory fluid flow regulates steady state osteopontin mRNA levels in a manner dependent on $Ca^{2+}_i$ mobilization and via extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways (9). In addition, other studies demonstrated that oscillations in intracellular $Ca^{2+}$ regulate expression of transcription factors such as NFAT, NFκB, and Oct/OAP, which may be involved in cellular differentiation (12, 13). Thus, understanding the molecular mechanism of fluid-induced $Ca^{2+}_i$ mobilization is a very important step elucidating mechanotransduction pathways in osteoblastic cells.

It is well documented that extracellular nucleotides such as ATP and UTP induce a broad spectrum of cell responses including $Ca^{2+}_i$ mobilization. These responses include excitation of sympathetic neurons, muscle cell proliferation, endothelial cell adhesion, spermiogenesis, acid-base equilibrium in intestinal epithelial cells, and mucociliary clearance in normal cystic fibrosis airway epithelia (14–16). It is also recognized that extracellular nucleotides act as important signaling molecules for cell-cell communication among bone cells (17). Additionally there is increasing evidence that extracellular nucleotides play

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[1] The abbreviations used are: FBS, fetal bovine serum; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); $Ca^{2+}_i$, intracellular calcium; MEM, minimal essential medium; $N/m^2$, Newtons/meter$^2$; P2Y2-AS, antisense oligodeoxynucleotides of P2Y2; P2Y2-SCR, scrambled control oligodeoxynucleotides of P2Y2.

an important role in bone remodeling (18). Therefore, extracellular nucleotides are potential candidates responsible for fluid flow-induced $Ca^{2+}{}_i$ mobilization in bone cells.

The extracellular nucleotides ATP and UTP are known to exert their effects via a family of specific receptors termed P2 purinergic receptors (purinoceptors) (19, 20). The P2 purinoceptors are divided into the P2X receptors, which are ligand-gated ion channels, and the P2Y receptors, which are G protein-coupled receptors. Up to seven P2X subtypes (P2X1-P2X7) and several types of P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11) have been cloned (19, 20). P2 purinoceptors are expressed in osteoblastic cells of rat and human origin (21), and several studies have demonstrated that extracellular nucleotides such as ATP and ADP increase osteoblastic cell proliferation and differentiation through specific P2 purinoceptors (22, 23).

Thus, in this study we hypothesized that extracellular nucleotides such as ATP and UTP and their specific purinoceptors mediate oscillatory fluid flow-induced $Ca^{2+}{}_i$ mobilization in osteoblastic cells. To test our hypothesis, we first utilized apyrase, an enzyme that quickly hydrolysis 5′ nucleotide triphosphates to monophosphates, to examine whether extracellular 5′ nucleotide triphosphates such as ATP and UTP are necessary for oscillatory fluid flow to induce $Ca^{2+}{}_i$ mobilization in MC3T3-E1 osteoblastic cells. Secondly, different nucleotides and their agonists or antagonists were added to the fluid medium to investigate the possible involvement of different nucleotides responsible for oscillatory fluid flow-induced $Ca^{2+}{}_i$ mobilization. We identified P2Y as the purinoceptors involved in the fluid flow-induced $Ca^{2+}{}_i$ response. Finally, we employed molecular techniques to manipulate functional purinoceptor expression in osteoblastic cells to confirm that a specific purinoceptor, P2Y2, is involved in oscillatory fluid flow-induced $Ca^{2+}{}_i$ responses.

## MATERIALS AND METHODS

*Cell Culture*—The mouse osteoblastic cell line MC3T3-E1 was cultured in minimal essential α medium (MEM-α) (Invitrogen) containing 10% FBS (Hyclone, Logan, UT) and 1% penicillin and streptomycin (Invitrogen). The rat osteoblastic cell line ROS 17/2.8, which does not normally express P2Y2 receptors (24), was grown in minimal essential medium (MEM) supplemented with 10% heat-inactivated calf serum (Hyclone), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate (Invitrogen), 2 mM L-glutamine, and 1% penicillin and streptomycin. ROS 17/2.8 cells transfected with human P2Y2 purinoceptors (ROS/P2Y2) were cultured in the same media as ROS 17/2.8 but with 400 μg/ml Geneticin (Invitrogen) added. All cells were maintained in a humidified incubator at 37 °C with 5% $CO_2$. $0.85 \times 10^5$ cells were subcultured on quartz slides for 2 days before experiments. The fluid flow chamber employed in this study is a parallel plate design, and the flow delivery device generated 1 Hz sinusoidally oscillating flow (peak shear stress 2 N/m$^2$). Both have been described in previous studies (9, 25).

*Calcium Imaging*—$[Ca^{2+}]_i$ was quantified with the fluorescent dye fura-2. Fura-2 exhibits a shift in absorption when bound to $Ca^{2+}$ such that the emission intensity when illuminated with ultraviolet light increases with $[Ca^{2+}]_i$ at a wavelength of 340 nm and decreases with $[Ca^{2+}]_i$ at 380 nm. The ratio of light intensity between the two wavelengths corresponds to $[Ca^{2+}]_i$. A calibration curve of intensity ratio and $[Ca^{2+}]_i$ was obtained using fura-2 in buffered calcium standards supplied by the manufacturer (Molecular Probes, Inc., Eugene, OR). Preconfluent (80%) cells were washed with MEM-α and 2% FBS at 37 °C, incubated with a 10 μM fura-2-acetoxymethyl ester (Molecular Probes) solution for 30 min at 37 °C, and washed again with fresh MEM-α and 2% FBS. The quartz slide with the cells was mounted on a parallel plate flow chamber filled with the experimental media. The chamber was placed on an inverted fluorescence microscope (Nikon, Melville, NY) and left undisturbed for 30 min before experiments. Cell ensembles were illuminated at wavelengths of 340 and 380 nm in turn. Emitted light was passed through a 510-nm interference filter and detected with an intensifier charge-coupled device camera (International LTD., Sterling, VA). Images were recorded once every 2 s and analyzed using image analysis software (Metafluor; Universal Imaging, West Chester, PA). Basal $[Ca^{2+}]_i$ was sampled for 3 min and followed by 3 min of oscillatory fluid flow.

*Oligodeoxynucleotide Treatment*—Antisense oligodeoxynucleotides directed against P2Y2, 5′-CAG GTC TGC TGC CAT-3′ (P2Y2-AS), which had been demonstrated to inhibit P2Y2 purinoceptor function in astrocytes (26), were employed to target the P2Y2 purinoceptor on MC3T3-E1 cells. Scrambled control oligodeoxynucleotides of P2Y2, 5′-GTG CTC GTA CGT ACC-3′ (P2Y2-SCR), were designed and synthesized at the Core Facilities of Pennsylvania State University College of Medicine. $8 \times 10^4$ MC3T3-E1 cells were precultured for 24 h and incubated in the presence of P2Y2-AS or P2Y2-SCR with LipofectAMINE PLUS (Invitrogen) for 2.5 h. After transfection, cells were cultured in fresh culture medium for another 24 h before fluid flow experiments. Cellular uptake of antisense oligodeoxynucleotides, which were labeled with fluorescein isothiocyanate at their 5′-end (Synthetic Genetics Inc., San Diego, CA), was verified by observation of fluorescein isothiocyanate with a fluorescence microscope.

*Pharmacological Agents*—Five nucleotides (ATP, UTP, GTP, CTP, and TTP) were employed as extracellular signaling molecules in flow experiments. Apyrase (10 units/ml), an enzyme that rapidly hydrolyzes 5′ nucleotide triphosphates to monophosphates, was used to inhibit the nucleotides effects (27) and was applied 30 min before fluid flow. Different purinoceptor agonists (ADP, UDP, ATPγS, and adenosine) and antagonist (pyridoxal phosphate 6-azophenyl-2′,4′-disulfonic acid) were added to the experimental media to identify which specific purinoceptors were expressed on the MC3T3-E1 cells. Pertussis toxin (Calbiochem) was used to assess the role of $G_{i/o}$-proteins in the $Ca^{2+}{}_i$ response to fluid flow. Cells were exposed to 200 ng/ml pertussis toxin in media for 90 min before fluid flow. To remove triphosphate contamination from diphosphate nucleotides, 1 mM stock solutions of UDP and ADP were pretreated with 10 units/ml hexokinase for 30 min at 37 °C in the presence of 5 mM glucose (28). All the reagents were from Sigma unless otherwise indicated.

*Data Analysis*—We used a numerical procedure from mechanical analysis known as Rainflow cycle counting to identify calcium oscillations (29). Briefly, this technique identifies complete cycles or oscillations in the time history data even when they are superimposed upon each other and, therefore, can be used to distinguish and quantify $Ca^{2+}{}_i$ responses from background noise. We defined a response as an oscillation in $[Ca^{2+}]_i$ at least 2-fold greater than that of the average base-line level of nontreated cells. Base-line $[Ca^{2+}]_i$ data were recorded for each slide for 3 min before the application of oscillatory fluid flow.

Data were expressed as the mean ± S.E. To compare observations from no flow and flow responses, a two-sample Student *t* test was used in which sample variance was not assumed to be equal. To compare observations from more than two groups, a one-way analysis of variance was employed followed by a Bonferroni selected-pairs multiple comparisons test (Instat, GraphPad Software Inc., San Diego, CA). $p < 0.05$ was considered statistically significant.

## RESULTS

*Extracellular Nucleotides Are Necessary for Fluid Flow-induced $Ca^{2+}{}_i$ Mobilization*—To investigate the role of extracellular molecules in FBS or induced by FBS in fluid flow-induced $Ca^{2+}{}_i$ mobilization, MC3T3-E1 cells were exposed to flow media with or without FBS (Fig. 1*A*). In the absence of 2% FBS, oscillatory fluid flow (2N/m$^2$, 1Hz) induced an increase in $[Ca^{2+}]_i$ in only 5.72 ± 1.44% of cells, a value not significantly different from that of cells during the no flow period (4.35 ± 1.31%) ($p > 0.05$) (Fig. 1*C*). However, within 30 s of starting oscillatory fluid flow with the media containing 2% FBS, 65.9 ± 3.94% of cells increased $[Ca^{2+}]_i$, a value significantly greater than the no flow control (5.96 ± 1.05%) ($p < 0.01$) (Figs. 1, *B* and *C*). In the presence of both 2% FBS and apyrase (10 units/ml), the percentage of cells responding with an increase in $[Ca^{2+}]_i$ was 3.91 ± 1.62%, a value not significantly different from the percentage responding during the no flow period (2.64 ± 1.98%) ($p > 0.05$). As was the case with our previous studies (9), the average amplitude of $[Ca^{2+}]_i$ in all responding cells during all oscillatory flow conditions was not significantly different from that of cells during no flow periods (data not shown).

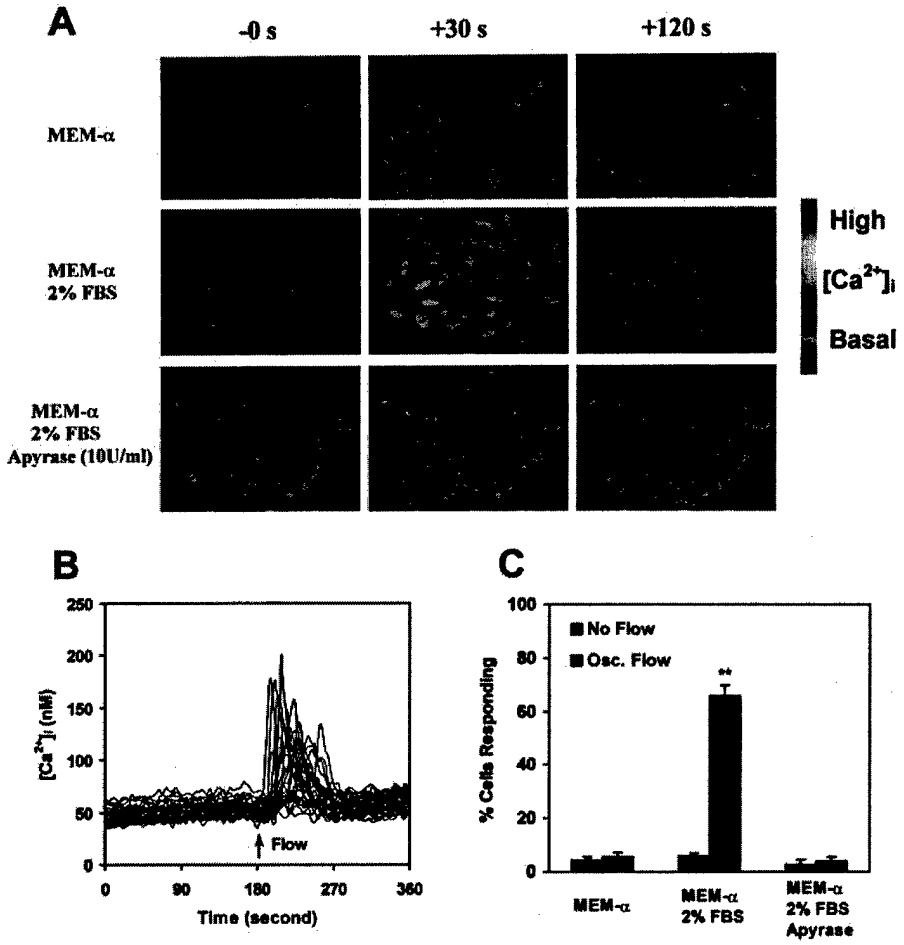


FIG. 1. **Oscillatory fluid flow induces increases in $[Ca^{2+}]_i$ in MC3T3-E1 osteoblastic cells.** *A*, monolayers of MC3T3-E1 cells were loaded with fura-2 then exposed to oscillatory fluid flow (2N/m², 1Hz) during fluorescence ration imaging. Times after stimulation in seconds is indicated on the *top*. The *top three images* are from cells exposed in MEM-α, and the *middle three images* are from cells in MEM-α/2%FBS. The *bottom images* are from cells in MEM-α, 2% FBS in the presence of apyrase (10 units/ml). The pseudocolor map represents calibrated $[Ca^{2+}]_i$. *B*, an example of MC3T3-E1 cell $[Ca^{2+}]_i$ traces obtained for oscillatory flow (2N/m², 1Hz) in MEM-α, 2% FBS. The *arrow* depicts the onset of flow, and each *line* represents an individual cell response. *C*, the percentage of MC3T3-E1 cells responding with an increase in $[Ca^{2+}]_i$ in the three flow media, MEM-α, MEM-α/2%FBS, and MEM-α, 2%FBS in the presence of apyrase (10 units/ml), respectively. In flow media of MEM-α, 2% FBS, the percentage of MC3T3-E1 cells responding to oscillatory (*Osc.*) flow with an increase in $[Ca^{2+}]_i$ was significantly different from that in the no flow period (**, $p < 0.01$). However, in MEM-α or MEM-α, 2%FBS in the presence of apyrase (10 units/ml), the percentage of MC3T3-E1 cells responding with an increase in $[Ca^{2+}]_i$ was not significantly different from that during no flow periods ($p > 0.05$). Each *bar* represents the mean ± S.E., each group of data was obtained from six individual experiments, and the three groups have a total of 309, 334, and 298 cells, respectively.

*ATP and UTP Are Involved in Fluid Flow-induced $Ca^{2+}{}_i$ Mobilization*—To examine whether extracellular nucleotide triphosphates are involved in fluid flow-induced $Ca^{2+}{}_i$ mobilization, all five nucleotide triphosphates (ATP, UTP, GTP, CTP, and TTP) were added individually to the fluid flow medium to replace 2% FBS. In the presence of 5 μM ATP or UTP, exposure to oscillatory fluid flow resulted in 74.3 ± 5.59 or 75.5 ± 2.28%, respectively, of cells displaying an increased $[Ca^{2+}]_i$, whereas 1.19 ± 0.51 or 0.96 ± 0.57% of cells did so during no flow periods ($p < 0.01$) (Fig. 2). However, only 4.19 ± 1.52, 5.38 ± 2.13, or 3.52 ± 2.16% of cells responded to oscillatory fluid flow with an increased $[Ca^{2+}]_i$ in the presence of CTP, GTP, TTP (5 μM), respectively, whereas 1.23 ± 1.0, 2.54 ± 1.64, or 1.23 ± 0.89%, respectively, of cells did so during control periods ($p > 0.05$).

*P2Y Purinoceptors Are Involved in Fluid Flow-induced $Ca^{2+}{}_i$ Mobilization*—To identify which purinoceptors are responsible for oscillatory fluid flow and, potentially, extracellular signaling molecule-induced $Ca^{2+}{}_i$ mobilization, purinoceptor agonists or antagonists were added to the flow medium. In the presence of adenosine (5–50 μM), a P1 purinoceptor agonist, oscillatory fluid flow did not induce increases in $[Ca^{2+}]_i$ in a greater percentage of cells than the oscillatory flow control (no FBS) (3.53 ± 1.92% *versus* 4.34 ± 1.45%; $p > 0.05$) (Fig. 3). This was also the case in the presence of ATPγS (5–50 μM), a P2X and P2Y11 purinoceptor agonist (6.39 ± 3.23% *versus* 4.34 ± 1.45%; $p > 0.05$). Moreover, neither the specific P2Y1 purinoceptor agonist ADP (5–50 μM) nor the P2Y6 purinoceptor agonist UDP (5–50 μM) was able to increase the percentage of cells responding to oscillatory fluid flow above the percentage responding to oscillatory flow control (no FBS) (2.46 ± 1.23 and 4.38 ± 2.13%, respectively, *versus* 4.34 ± 1.45%; $p > 0.05$). In the presence of 2% FBS, pyridoxal phosphate 6-azophenyl-2′,4′-disulfonic acid (*PPADS*) (30 μM), a P2X antagonist, did not have any effect on the fluid flow-induced $Ca^{2+}{}_i$ response rela-

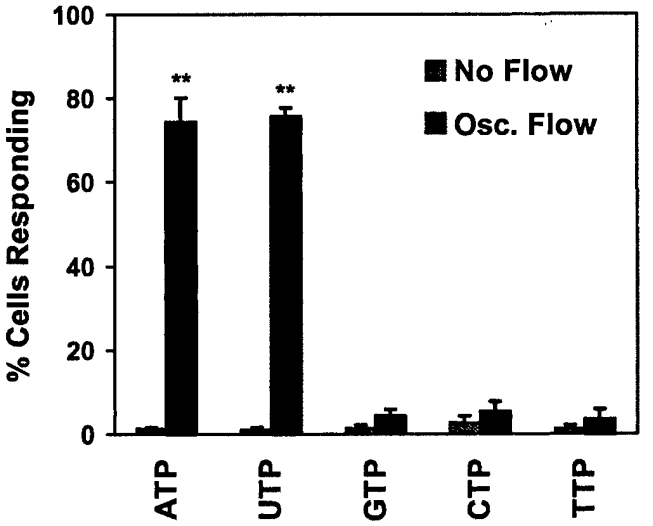


FIG. 2. **Two of the five nucleotide triphosphates, ATP and UTP, are able to restore the ability of oscillatory fluid flow to increase $[Ca^{2+}]_i$ in the absence of FBS.** The graph depicts the percentage of MC3T3-E1 cells responding to oscillatory flow (2N/m², 1Hz) with an increase in $[Ca^{2+}]_i$ in MEM-α with ATP, UTP, GTP, CTP, or TTP added, individually, to replace 2% FBS. Only with added ATP or UTP was the percentage of cells responding to oscillatory (*Osc.*) flow with an increase in $[Ca^{2+}]_i$ significantly different from that in the no flow period (**, $p < 0.01$). Each *bar* represents the mean ± S.E., and each experiment was repeated on 4–6 slides.

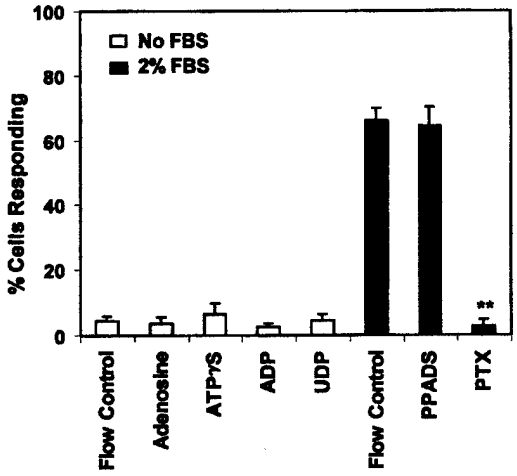


FIG. 3. **Effect of purinoceptor agonists and antagonists on oscillatory fluid flow induced $Ca^{2+}{}_i$ mobilization.** The graph depicts the percentage of MC3T3-E1 cells responding to oscillatory (*Osc.*) flow (2N/m², 1Hz) with an increase in $[Ca^{2+}]_i$ in the two flow media, MEM-α (*No FBS*; *opened bars*) and MEM-α/2%FBS (2% FBS; *closed bars*). When either adenosine, ATPγS, ADP, or UDP (5–50 μM) were added to the MEM-α, the percentage of cells responding to oscillatory flow displaying an increase in $[Ca^{2+}]_i$ was not significantly different from that in the flow control (*No FBS*) ($p > 0.05$). Pyridoxal phosphate 6-azophenyl-2',4'-disulfonic acid (*PPADS*) (30 μM), a P2X antagonist, had no effect on the oscillatory fluid flow-induced $Ca^{2+}{}_i$ response relative to the flow control (2% FBS) ($p > 0.05$). However, pertussis toxin (*PTX*; 200 ng/ml), which inhibits the $G_{i/o}$ protein, to which P2Y receptors but not other purinoceptors are coupled, dramatically reduced fluid flow-induced increases in $[Ca^{2+}]_i$ (**, $p < 0.01$, *versus* flow control with 2% FBS). Each *bar* represents the mean ± S.E., and each experiment was repeated on 4–6 slides.

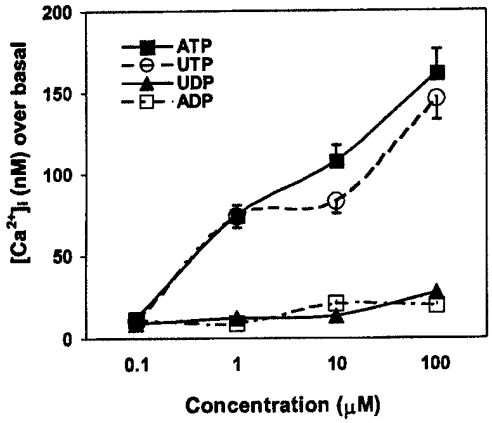


FIG. 4. **MC3T3-E1 cells express P2Y2 purinoceptors.** Fura-2-loaded MC3T3-E1 cells were perfused with a variety of nucleotides agonists over a concentration range of 0.1–100 μM (as indicated on the *x* axis). The average rise in $[Ca^{2+}]_i$ was ATP, UTP, ADP, and UDP concentration-dependent. Each data point represents the mean ± S.E. of the response to applied agonists at each concentration, $n = 35–69$ cells.

tive to the oscillatory flow control (2% FBS) (64.3 ± 5.85 *versus* 65.9 ± 3.94%; $p > 0.05$). However, pertussis toxin (*PTX*; 200 ng/ml), which inhibits $G_{i/o}$ proteins, to which P2Y purinoceptors but not other purinoceptors are coupled dramatically reduced the percentage of cells responding to fluid flow (in the presence of 2% FBS) relative to the oscillatory flow control (in the presence of 2% FBS) (2.67 ± 2.16 *versus* 65.9 ± 3.94%; $p < 0.01$).

*P2Y2 Purinoceptors Are Involved in Fluid Flow-induced $Ca^{2+}{}_i$ Mobilization*—Our agonist and antagonist results suggested that P2Y purinoceptors contribute to oscillatory fluid flow-induced increases in $[Ca^{2+}]_i$. Therefore, we examined the function of P2 purinoceptors in MC3T3-E1 osteoblastic cells. The dependence of the rise in $[Ca^{2+}]_i$ on ATP, UTP, ADP, and UDP concentration was assessed (Fig. 4), and the equipotency of ATP and UTP suggested P2Y2 purinoceptor expression in the MC3T3-E1 cells. To further confirm that P2Y2 purinoceptors are involved in fluid flow-induced $Ca^{2+}{}_i$ mobilization, MC3T3-E1 cells were exposed to fluid flow in the presence of mouse P2Y2 antisense oligodeoxynucleotides. ATP (5 μM) increased $[Ca^{2+}]_i$ in P2Y2-SCR-treated MC3T3-E1 cells but did so to a lesser degree in P2Y2-AS-treated cells (74.9 ± 3.36 nM *versus* 136.4 ± 6.29 nM, respectively, $p < 0.01$) (Fig. 5*A*). P2Y2-AS treatment significantly decreased the percentage of MC3T3-E1 cells responding to fluid flow with an increase in $[Ca^{2+}]_i$ relative to P2Y2-SCR-treated cells (29.0 ± 9.03% *versus* 65.3 ± 7.69%; $p < 0.05$) in the presence of 2% FBS (Fig. 5*B*). We next examined oscillatory fluid flow responsiveness in ROS 17/2.8 osteoblastic cells, which normally do not express P2Y2 purinoceptors, genetically engineered to express P2Y2 purinoceptors (24). Oscillatory fluid flow induced an increase in $[Ca^{2+}]_i$ in significantly fewer wild type ROS 17/2.8 cells than in P2Y2 purinoceptors expressing ROS 17/2.8 cells (25.80 ± 6.0 *versus* 69.0 ± 5.87%; $p < 0.01$) (Fig. 6).

## DISCUSSION

Although it is clear mechanical loading plays an important role in bone remodeling at the tissue level, little is known about the cellular and molecular mechanisms of bone cell mechanotransduction (2, 3). Accumulating evidence suggests that extracellular fluid flow induced by mechanical loading is a potential physiological signal to trigger osteoblastic cell response to mechanical loading and may be a better candidate for transducing mechanical loads than other biophysical signals including substrate deformation and endogenous electric fields (6–8, 30). Recently, we demonstrated that oscillatory fluid flow, which mimics fluid flow induced by mechanical loading *in vivo*, is a potent bone cell activator and induces $Ca^{2+}{}_i$ release and increases osteopontin gene expression via extracellular signal-regulated kinase 1/2 and p38 activation (9). Additionally the increased osteopontin expression is $Ca^{2+}{}_i$-dependent. However, the molecular mechanisms of fluid flow-induced $Ca^{2+}{}_i$ mobilization are still unknown. Our findings are the first direct experimental evidence demonstrating that oscillatory fluid flow and the extracellular signaling molecules ATP and UTP, interacting with their specific P2Y purinoceptors, play an important role in bone cell mechanotransduction.

Our data demonstrate that extracellular signaling molecules contribute to oscillatory fluid flow-induced $Ca^{2+}{}_i$ mobilization since oscillatory fluid flow did not activate $Ca^{2+}{}_i$ mobilization in the absence of the extracellular signaling molecules present in FBS. This is consistent with prior studies that demonstrate that FBS modulates the $Ca^{2+}{}_i$ response of primary cultured bone cells to steady fluid flow (10). In the absence of FBS steady fluid flow only minimally and inconsistently induced $Ca^{2+}{}_i$ mobilization in primary osteoblastic cells.

Several lines of evidence suggest that ATP is involved in mechanically induced $Ca^{2+}{}_i$ mobilization. For instance, steady fluid flow modulates the $Ca^{2+}{}_i$ response to ATP in vascular endothelial cells (31). Additionally, exposure of rat aortic smooth muscle cells to mechanical stretch (32) and rabbit endothelial cells (33) as well as human astrocytoma cells (34) to fluid flow stimulates the release of ATP. In skeletal tissue ATP induces osteoblastic cell $Ca^{2+}{}_i$ mobilization (35), and hydrostatic pressure stimulates ATP release from the chondocytes in pellet cultures. Finally, the addition of apyrase, which rapidly hydrolyzes 5′ nucleotide triphosphates to monophosphates, prevents mechanically induced $Ca^{2+}{}_i$ wave propagation in polarized epithelia cultures (27). Taken together, these results

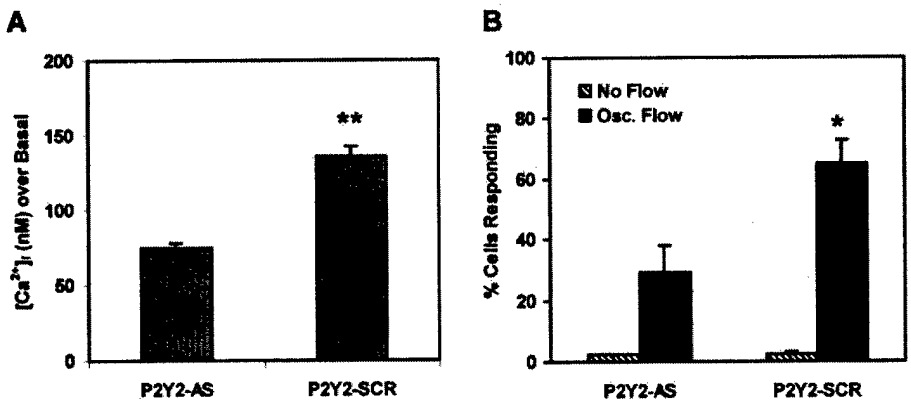


FIG. 5. **P2Y2-AS treatment inhibits oscillatory fluid flow induced increases in $[Ca^{2+}]_i$ in MC3T3-E1 osteoblastic cells.** *A*, when MC3T3-E1 cells were exposed to 5 $\mu$M ATP, the average increase in $[Ca^{2+}]_i$ amplitude over basal in P2Y2-AS-treated cells was significantly reduced relative to P2Y2-SCR treated cells (**, $p < 0.01$). Each data point represents the mean ± S.E. of the response to applied agonists at each concentration, $n$ = 77–79 cells. *B*, P2Y2-AS treatment significantly decreased the percentage of MC3T3-E1 cells responding to oscillatory (*Osc.*) fluid flow (2N/m$^2$, 1 Hz) with an increase in $[Ca^{2+}]_i$ relative to P2Y2-SCR-treated cells in the presence of 2% FBS (*, $p < 0.05$). Each *bar* represents the mean ± S.E., and each experiment was repeated on six slides.

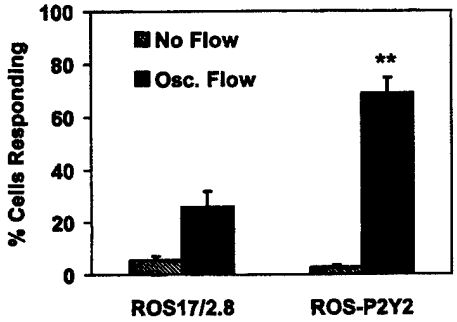


FIG. 6. **Exposure of oscillatory fluid flow increases $[Ca^{2+}]_i$ in ROS 17/2.8 cells expressing the P2Y2 purinoceptors.** Oscillatory (*Osc.*) fluid flow (2N/m$^2$, 1Hz) induced an increase in $[Ca^{2+}]_i$ in significantly fewer wild type ROS 17/2.8 cells than in P2Y2 expressing ROS 17/2.8 cells (**, $p < 0.01$). Each *bar* represents the mean ± S.E., and each experiment was repeated on six slides.

suggest that extracellular nucleotides such as ATP may contribute to oscillatory fluid flow-induced $Ca^{2+}{}_i$ mobilization in osteoblastic cells.

Our results also demonstrate that two of the five nucleotide triphosphates, ATP and UTP, are able to restore the ability of fluid flow to increase $[Ca^{2+}]_i$ in the absence of FBS, suggesting that ATP and UTP may mediate the effect of fluid flow on $[Ca^{2+}]_i$. Previous studies demonstrate that exposure of endothelial cells to steady fluid flow in the presence of ATP induces $Ca^{2+}{}_i$ mobilization (31, 36). Additionally, it has been demonstrated that UTP is involved in the intracellular $Ca^{2+}$ wave propagation induced by mechanical stimulation in polarized epithelia (27). Thus, our data as well as other studies suggest ATP and UTP are involved in mechanically induced $Ca^{2+}{}_i$ mobilization.

In this study, we demonstrated that P2Y receptors (P2Y2 or P2Y4) may be involved in the $Ca^{2+}{}_i$ response to fluid flow by adding different purinoceptor agonists and antagonists to the flow media. Furthermore, treatment with P2Y2 antisense oligodeoxynucleotides decreased the percentage of MC3T3-E1 osteoblastic cells that respond to fluid flow with an increase in $[Ca^{2+}]_i$. Additionally, ROS 17/2.8 cells, which do not normally express P2Y2 purinoceptors, transfected with P2Y2 purinoceptors responded to fluid flow in the presence of 2% FBS, confirming the involvement of P2Y2 purinoceptors. Taken together, our data suggest that P2Y2 purinoceptors are responsible for oscillatory fluid flow-induced $Ca^{2+}{}_i$ mobilization in osteoblastic cells. Similarly, P2Y2 purinoceptors have been demonstrated to be involved in mechanically induced $Ca^{2+}{}_i$ wave propagation in airway epithelia *in vitro* (27).

Although our antisense treatment of MC3T3-E1 cells significantly decreased the percentage of cells responding with an increases in $[Ca^{2+}]_i$, 29.0 ± 9.03% of the cells still responded to oscillatory fluid flow. Furthermore, antisense treatment only partially inhibited ATP-induced $Ca^{2+}{}_i$ mobilization. This suggests that P2Y2 purinoceptors were still being expressed after antisense treatment. Previous studies demonstrated that wild type ROS 17/2.8 cells, which lack P2Y2 purinoceptors, do not respond to steady fluid flow or ATP with an increase in $[Ca^{2+}]_i$ (21, 37). On the other hand, we found that oscillatory fluid flow induced an increase in $[Ca^{2+}]_i$ in a very low percentage of ROS 17/2.8 cells, just over 25.8% of the cells exposed. The difference of our study and others may be due to a different source of ROS 17/2.8 cells and culture environment. It may also suggest that the other P2 purinoceptors expressed in ROS 17/2.8 cells may contribute to fluid flow-induced $Ca^{2+}{}_i$ responses.

Although our data suggest that P2Y purinoceptors are responsible for oscillatory fluid flow-induced $Ca^{2+}{}_i$ mobilization in osteoblastic cells, other studies demonstrated that in human endothelial cells steady fluid flow in the presence of ATP activates $Ca^{2+}{}_i$ influx via P2X purinoceptors (36). This suggests that either endothelial cells utilize different purinoceptors for mechanotransduction or that steady fluid flow utilizes a different mechanism than oscillatory fluid flow. This emphasizes the importance of using a mechanical stimulus appropriate for the cells being examined when trying to elucidate mechanotransduction pathways. Furthermore, that endothelial cells may utilize different purinoceptors in mechanotransduction than do osteoblastic cells suggests that osteoblastic mechanotransduction pathways can be targeted, *e.g.* in developing bone anabolic agents, without affecting endothelial cell mechanotransduction.

The physiological consequence of mechanically induced activation of the ATP/cytosolic $Ca^{2+}$ pathway in osteoblastic cells is unclear. However, ATP, through an interaction with P2X purinoceptors, activates DNA synthesis in human osteoblastic MG-63 cells (23). Additionally ATP, acting through P2Y purinoceptors, modulates mesenchymal cell differentiation *in vitro* (38). These observations together with data suggesting that mechanical signals, including fluid flow, increase the expression of markers of osteoblastic differentiation (6, 39–44) suggest that extracellular nucleotides may mediate the effect of mechanical signals, especially fluid flow, on bone cell differentiation.

Several limitations should be considered when interpreting our results. For instance, although our results strongly suggest that P2Y2 purinoceptors are involved in fluid flow-induced $Ca^{2+}{}_i$ mobilization, it is also likely the other P2Y purinoceptors, for instance P2Y4 purinoceptors, may be involved in $Ca^{2+}{}_i$ mobilization. Therefore, the comprehensive characterization of all P2Y subtypes of osteoblastic cells and their roles in bone cell

mechanotransduction is under way in our laboratory. From this study, it is still not clear whether extracellular nucleotides (ATP, UTP alone, or both) are released by cells after the cells sense oscillatory fluid flow or whether these nucleotides already exist locally around cells and are activated by fluid flow to induce $Ca^{2+}_i$ mobilization. Further investigation of the dynamics of the extracellular nucleotide release during fluid flow is necessary. Finally, our P2Y purinoceptor results were conducted in two different osteoblastic cell lines. Therefore, it is likely that the results can be extrapolated to other osteoblastic cell lines including primary cultures.

In summary, we have shown that extracellular nucleotides ATP and UTP are essential for oscillatory fluid flow-induced $Ca^{2+}_i$ mobilization in osteoblastic cells in the presence of FBS. Furthermore, P2Y, specifically P2Y2, is the purinoceptor responsible for oscillatory fluid flow-induced $Ca^{2+}_i$ mobilization. Taken together, these data suggest that oscillatory fluid flow acts through the interaction of the extracellular nucleotides ATP/UTP with P2Y (P2Y2 or P2Y4) purinoceptors to induce $Ca^{2+}_i$ mobilization in osteoblastic cells. Our studies shed new light of the molecular mechanisms responsible for oscillatory fluid flow induced $Ca^{2+}_i$ mobilization in osteoblastic cells. The understanding of molecular mechanisms of fluid flow-induced $Ca^{2+}_i$ mobilization in osteoblastic cells will provide guidance in developing novel therapeutic approaches to various diseases such as disuse osteopenia and age-related osteoporosis.

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# Flow-induced calcium oscillations in rat osteoblasts are age, loading frequency, and shear stress dependent

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**Donahue, Seth W., Christopher R. Jacobs, and Henry J. Donahue.** Flow-induced calcium oscillations in rat osteoblasts are age, loading frequency, and shear stress dependent. *Am J Physiol Cell Physiol* 281: C1635–C1641, 2001.—Bone adaptation to mechanical loading is dependent on age and the frequency and magnitude of loading. It is believed that load-induced fluid flow in the porous spaces of bone is an important signal that influences bone cell metabolism and bone adaptation. We used fluid flow-induced shear stress as a mechanical stimulus to study intracellular calcium ($Ca_i^{2+}$) signaling in rat osteoblastic cells (ROB) isolated from young, mature, and old animals. Fluid flow produced higher magnitude and more abundant $[Ca^{2+}]_i$ oscillations than spontaneous oscillations, suggesting that flow-induced $Ca_i^{2+}$ signaling encodes a different cellular message than spontaneous oscillations. ROB from old rats showed less basal $[Ca^{2+}]_i$ activity and were less responsive to fluid flow. Cells were more responsive to 0.2 Hz than to 1 or 2 Hz and to 2 Pa than to 1 Pa. These data suggest that the frequency and magnitude of mechanical loading may be encoded by the percentage of cells displaying $[Ca^{2+}]_i$ oscillations but that the ability to transduce this information may be altered with age.

mechanotransduction; osteoblast; calcium signaling; bone adaptation

BONES ADAPT to mechanical loading. When normal mechanical loading is absent, bone mass is removed. For example, disuse osteopenia occurs in the tibias of astronauts who experience microgravity (49), in patients confined to prolonged bedrest (28), in immobilized bones following surgery (30), and in patients with total arthroplasty (29). When habitual bone loading is exceeded, bone mass is added. For instance, periosteal and endosteal bone areas have been found to be significantly higher in the dominant arm of tennis players (3). Bone mineral density and cross-sectional moment of inertia have been found to be significantly higher in the dominant humeri of tennis players, regardless of the age at which they started playing (16). However, the effect of mechanical loading on bone mass was more than twofold greater in young players than in players who began playing after reaching skeletal maturity. These data suggest that growing bones are more adaptable to mechanical loading than adult bones. Turner et al. (44–46) have demonstrated the ability of rat long bones to adapt to unaccustomed mechanical loading. They showed that new bone formation in tibias loaded in four-point bending was dependent on the frequency and magnitude of loading (44, 45). They also demonstrated that the ability of long bones to adapt to mechanical loading was diminished in 19-mo-old rats compared with 9-mo-old rats (46). At the highest bending load, the relative bone formation rate was more than 16-fold lower in the older rats. These findings parallel human studies, which suggest that cells in growing bones are more sensitive to mechanical signals than cells in adult bones.

It is believed that physical activities, which produce bending loads in bones, induce fluid flow in the porous spaces of bone (10, 15, 26, 27, 50). This fluid flow is believed to be an important physical signal that influences bone cell metabolism and bone adaptation to mechanical loading (7, 15, 27). Bone cells produce adaptations to mechanical loading: osteoblasts add bone mass when loading becomes excessive, and osteoclasts remove unneeded bone. However, the biochemical signaling pathways that mediate bone adaptation to mechanical loading are unknown. Understanding how individual bone cells respond to mechanical stimuli with biochemical responses and how these responses change with age may help elucidate our understanding of mechanically induced bone adaptations and the etiology of bone diseases such as osteoporosis.

In vitro, physical stimuli activate numerous signaling molecules in bone cells, including intracellular calcium (9, 17, 18, 51, 52, 55), prostaglandins (1, 36, 40), inositol trisphosphate (36), and nitric oxide (25, 32, 40). Mechanical stimuli also have been shown to upregu-

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late gene expression in bone cells (9, 20, 54). Intracellular calcium oscillations are important signaling mechanisms for many cellular processes (e.g., differentiation, proliferation, and gene transcription) (5). Calcium signaling also is an early response in bone cell mechanotransduction and can influence downstream signaling events. For example, blocking mechanically induced intracellular calcium oscillations also blocks gene expression (9, 54), prostaglandin release (1), and cytoskeletal reorganization (9).

In vivo rat studies have demonstrated that long bone adaptation to mechanical loading is magnitude and frequency dependent and that the capacity for adaptation decreases with age (44–46). Therefore, it is likely that biochemical signaling in bone cells in response to mechanical stimuli is also dependent on age and on the magnitude and frequency of the physical signal. We hypothesized that fluid flow-induced oscillations in cytosolic calcium concentration ($[Ca^{2+}]_i$), in osteoblastic cells isolated from rat long bones, would be dependent on loading frequency, shear stress magnitude, and age of the rat from which the cells were isolated. Biochemical messages encoded by $[Ca^{2+}]_i$ oscillations may be determined by the magnitude and/or frequency of the oscillation (6, 43). It also is thought that calcium signaling requires coordinated $Ca_i^{2+}$ signaling events in cell ensembles.(21) Therefore, we chose the percentage of cells displaying $[Ca^{2+}]_i$ oscillations and the magnitude of the oscillations as the independent response variables to fluid flow-induced shear stress.

## METHODS

*Bone cells.* Rat osteoblastic cells (ROB) were isolated from the humeri, tibias, and femora of young (4 mo, $n = 7$), mature (12 mo, $n = 7$), and old (24 mo, $n = 7$) male Fisher 344 rats. All procedures were approved by the Institutional Animal Care and Use Committee at the M. S. Hershey Medical Center. The rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (Nembutal; Abbott Laboratories, North Chicago, IL) with a dosage of 50 mg/kg of bodyweight and were euthanized by exsanguination. The bones were extracted from the animals, and subperiosteal ROB were obtained by removing all soft tissues, including cartilage and periosteum, from the bones and performing sequential collagenase (Worthington Biochemical, Lakewood, NJ) digestions at 37°C. Cells from the first digestion were collected by centrifugation and discarded to eliminate any residual non-bone cells that were not removed by dissection. Cells from the second digestion were collected by centrifugation and grown to confluency in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Gaithersburg, MD), 20% fetal bovine serum (FBS), and 1% penicillin/streptomycin. Cells from all six bones were pooled and grown to confluency in the same culture flask. We have shown previously that ROB isolated by this technique display characteristics of the osteoblast phenotype (13). We also stained ROB for alkaline phosphatase activity and performed a dye transfer assay to demonstrate gap junctional intercellular communication (53).

Three days before experimentation, the cells were plated on quartz microscope slides (76 × 26 × 1.6 mm) at a density of 75,000 cells per slide; cells were ~70% confluent on the day of experimentation. The cells were incubated at 37°C with 10 μM fura 2-AM (Molecular Probes, Eugene, OR) for 30 min before mechanical stimulation.

*Fluid flow system.* After fura 2 loading, the cell-seeded microscope slides were mounted in a parallel-plate flow chamber that was fixed to the stage of a fluorescent microscope. A fresh bolus of flow medium was added to the chamber, and the cells were left undisturbed for 30 min. The flow medium consisted of DMEM and 2% FBS. We have described previously the fluid flow system in detail (18); it will be described briefly here. To generate fluid flow-induced shear stresses on the cells in the chamber, a material-testing machine was used to pump a syringe, which was in series with rigid wall tubing and a flowmeter (Transonic Systems, Ithaca, NY), driving fluid through the chamber. This system produces laminar fluid flow in the chamber with an oscillating flow profile. Shear stresses on the chamber walls are proportional to the chamber dimensions and the rate of fluid flow (17). Thus we were able to generate shear stresses on the cells with magnitudes that they are predicted to experience in vivo (50).

Oscillating fluid flow was used because it more closely simulates physiological bone loading than steady or pulsatile flow (18). During experimentation, the cells were exposed to 3 min of oscillating fluid flow that produced shear stresses of 1 or 2 Pa at frequencies of 0.2, 1, or 2 Hz. Six slides of cells from each rat were randomly assigned to one of the six shear stress/frequency combinations.

*Calcium imaging.* Real-time $[Ca^{2+}]_i$ was quantified by using ratiometric dye methodology. When fura 2 binds $Ca^{2+}$, its maximal absorption wavelength shifts from 363 nm for $Ca^{2+}$-free fura 2 to 335 nm for $Ca^{2+}$-bound fura 2 (41). In practice, wavelengths of 340 and 380 nm are used for ratiometric measurements. The emission peak is near 510 nm for both $Ca^{2+}$-free and $Ca^{2+}$-bound fura 2. ROB ensembles were illuminated at wavelengths of 340 and 380 nm, emitted light was passed through a 510-nm filter, and images were collected with a charge-coupled device camera. Images of fluorescence intensities were collected every 2 s for a 3-min no-flow period (baseline) and for 3 min of oscillating fluid flow. $[Ca^{2+}]_i$ was determined from the ratio of the two emission intensities by using calibrated standards and image analysis software (Metaflour, West Chester, PA). Temporal $[Ca^{2+}]_i$ profiles were determined for 25–35 individual cells for each slide (Fig. 1).

Resting $[Ca^{2+}]_i$ was typically ≤50 nM in ROB. We defined a responsive cell as one that displayed a transient increase in $[Ca^{2+}]_i$ of at least 50 nM, because this represented at least a 100% increase over baseline. A numerical method known as Rainflow cycle counting was used to determine the magnitude of the calcium oscillations (19). We assessed the percentage of cells responding with a calcium oscillation and the magnitude of the responses.

*Statistics.* A factorial ANOVA was used to assess the influence of age, loading frequency, and shear stress on the percentage of cells responding to fluid flow with calcium oscillations and on the magnitude of the oscillations. ANOVAs were followed by Tukey's test for multiple mean comparisons. One-way ANOVAs were used to look for age-related differences for each shear stress/frequency combination. Use of ANOVA models requires the error terms to be normally distributed and requires constant variance for all factor levels (33). Studentized residuals were used to diagnose the validity of the model's assumptions. Frequency distributions of the residuals were used to check for outliers and normality of error terms. Plots of the residuals against fitted values were used to assess constancy of variance. Paired $t$-tests were used to compare the magnitudes of spon-

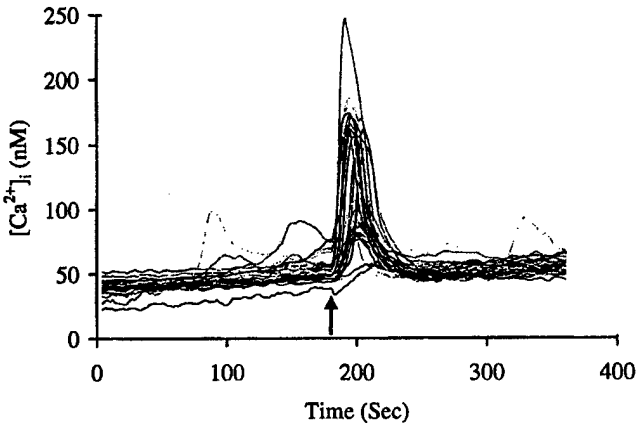


Fig. 1. Representative cytosolic calcium concentration ($[Ca^{2+}]_i$) profiles of 35 individual cells for a 3-min baseline period and 3 min of exposure to oscillating fluid flow [2 Pa, 1 Hz; rat osteoblastic cells (ROB) from a young rat]. Arrow indicates when flow was initiated. There were spontaneous $[Ca^{2+}]_i$ oscillations in the baseline period, and with the onset of fluid flow there was a greater and more coordinated response.

taneous and fluid flow-induced $[Ca^{2+}]_i$ oscillations in cells that had responses in both periods. A significance level of 0.05 was used for all statistical analyses.

## RESULTS

ROB from all three age groups displayed abundant alkaline phosphatase staining in confluent cultures. Our laboratory has demonstrated previously (12) that confluent cultures of ROB from young, mature, and old animals display highly functional gap-junctional intercellular communication. We found comparable functional communication in subconfluent ROB, from all three age groups, that were seeded on quartz microscope slides (not shown). These data provide further verification that ROB display characteristics of the osteoblast phenotype.

Individual ROB demonstrated one of four $[Ca^{2+}]_i$ profiles over the 6-min imaging period: *1*) spontaneous oscillation in the baseline period and no oscillation in the flow period, *2*) oscillations in both the baseline and flow periods, *3*) no oscillation in the baseline period and an oscillation in the flow period, and *4*) no oscillations in either period (Fig. 1). Rarely, cells displayed multiple oscillations in the baseline or flow periods. For the cells that did so, peak oscillations were used for statistical analyses. $[Ca^{2+}]_i$ oscillations in both periods typically lasted 60 s and returned to near-baseline values.

There were spontaneous $[Ca^{2+}]_i$ oscillations of at least 50 nM in ROB from all three age groups during the no-flow period. Of all the young ROB that were analyzed, 10% displayed spontaneous $[Ca^{2+}]_i$ oscillations. Significantly ($P = 0.04$), fewer ROB from old rats displayed spontaneous $[Ca^{2+}]_i$ oscillations during the no-flow period (Fig. 2*A*). However, there were no significant ($P = 0.17$) differences in the magnitude of the $[Ca^{2+}]_i$ oscillations among the three age groups (Fig. 2*B*).

With the onset of fluid flow, there were immediate and transient increases in $[Ca^{2+}]_i$ that lasted ~60 s (Fig. 1). Peak values were reached ~15 s after the onset of fluid flow. Significantly ($P < 0.0001$), more cells displayed $[Ca^{2+}]_i$ oscillations during the fluid-flow period than during the baseline period. Age ($P = 0.008$), loading frequency ($P = 0.0001$), and shear stress ($P = 0.035$) significantly influenced the percentage of cells responding to fluid flow. Mature ROB were more responsive than old ROB (Fig. 3). Cells were more responsive to 0.2 Hz than to 1 or 2 Hz (Fig. 4) and to 2 Pa than 1 Pa (Fig. 5). However, the magnitude of fluid flow-induced $[Ca^{2+}]_i$ oscillations was not significantly ($P = 0.367$) affected by age, loading frequency, or shear stress magnitude. The magnitude (mean ± SD) of the fluid flow-induced $[Ca^{2+}]_i$ oscillations, for all six loading regimes pooled, were 113 ± 60 nM for young ROB, 139 ± 102 nM for mature ROB, and 116 ± 85 nM for old ROB.

When the subpopulation of cells that displayed $[Ca^{2+}]_i$ oscillations in both the baseline and fluid-flow periods were considered, the magnitude of the fluid flow-induced $[Ca^{2+}]_i$ oscillations were significantly ($P < 0.0004$) larger than the magnitude of the sponta-

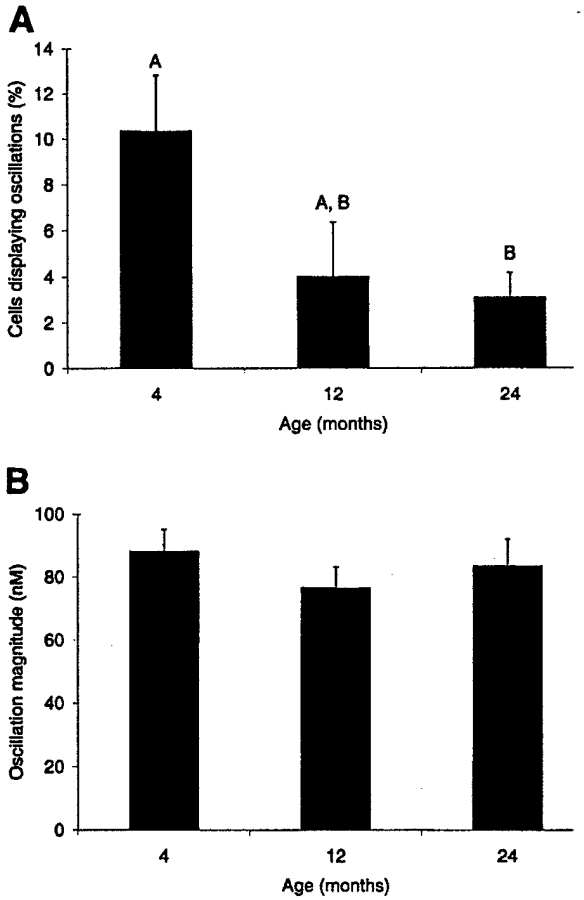


Fig. 2. *A*: influence of age on the percentage of cells displaying spontaneous $[Ca^{2+}]_i$ oscillations during the baseline period. Values are means with SE bars ($n = 42$ slides for each age group). Groups with the same letter (A, B) were not significantly different from each other. Cells from young rats showed significantly more oscillations than cells from old rats. *B*: magnitudes of spontaneous $[Ca^{2+}]_i$ oscillations in the baseline period were not significantly different among age groups.

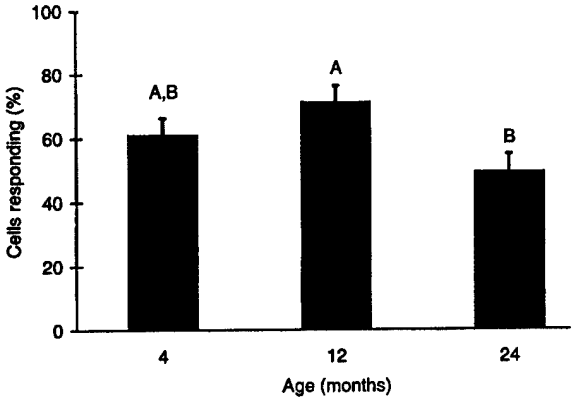


Fig. 3. Influence of age on the percentage of cells displaying $[Ca^{2+}]_i$ oscillations during the fluid-flow period. Values are means of all 6 loading regimes with SE bars ($n$ = 42 slides for each age group). Groups with the same letter (A, B) were not significantly different from each other. A significantly higher percentage of cells from mature rats responded to fluid flow than cells from old rats.

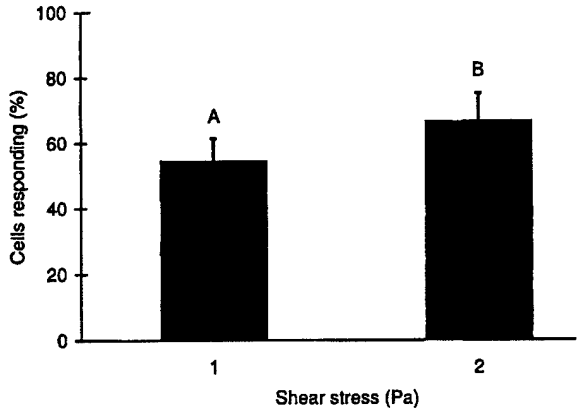


Fig. 5. Influence of shear stress on the percentage of cells displaying $[Ca^{2+}]_i$ oscillations during the fluid-flow period. Values are means of all age groups with SE bars ($n$ = 63 slides for each shear stress). Shear stress of 2 Pa was significantly more stimulatory than shear stress of 1 Pa.

neous $[Ca^{2+}]_i$ oscillations for all three age groups. In this subpopulation, the magnitude of the fluid flow-induced $[Ca^{2+}]_i$ oscillations were 58% greater than spontaneous $[Ca^{2+}]_i$ oscillations in young ROB, 134% higher in mature ROB, and 81% higher in old ROB. Of the cells that displayed spontaneous $[Ca^{2+}]_i$ oscillations in the baseline period, 80% of young ROB, 75% of mature ROB, and 76% of old ROB also exhibited flow-induced oscillations. Fluid flow was even able to potentiate the magnitude of the $[Ca^{2+}]_i$ oscillation in cells that were displaying spontaneous oscillations at the onset of fluid flow (Fig. 6).

The age of the rat from which cells were isolated significantly affected the percentage of cells responding to fluid flow in the factorial model. A significantly ($P$ = 0.008) larger percentage of ROB from mature rats (71%) had $[Ca^{2+}]_i$ oscillations than did ROB from old rats (49%) (Fig. 3). However, when each loading regime was considered separately, age did not significantly ($P > 0.141$) influence the percentage of cells responding to fluid flow at either 1 Pa (Fig. 7*A*) or 2 Pa (Fig. 7*B*).

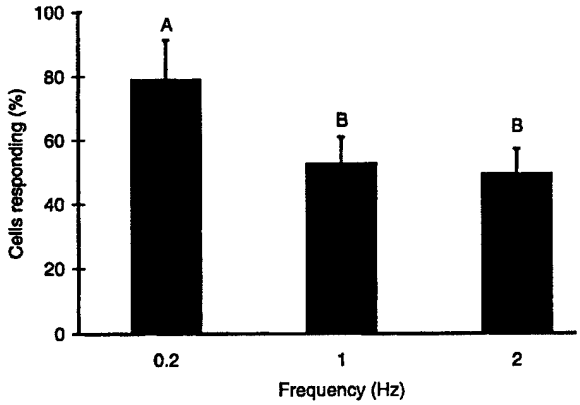


Fig. 4. Influence of loading frequency on the percentage of cells displaying $[Ca^{2+}]_i$ oscillations during the fluid-flow period. Values are means of all age groups with SE bars ($n$ = 42 slides for each frequency). A frequency of 0.2 Hz was significantly more stimulatory than a frequency of 1 or 2 Hz.

For each shear stress/frequency combination, however, old ROB were always the least responsive.

## DISCUSSION

Bones adapt to mechanical loading in a frequency- and magnitude-dependent fashion (44, 45). However, the bones of mature rats adapt better to unaccustomed mechanical loading than do the bones of old rats (46). It is widely believed that bone cells mediate bone adaptations to mechanical loading by activating signaling pathways that regulate bone modeling and remodeling (7, 8, 11, 15). We found that $Ca_i^{2+}$ signaling in ensembles of osteoblastic cells was dependent on the frequency and magnitude of a mechanical stimulus. In addition, we found that ensembles of ROB from mature rats were more responsive to fluid flow than were ROB from old rats.

$[Ca^{2+}]_i$ oscillations are involved in many normal cellular processes such as proliferation and gene ex-

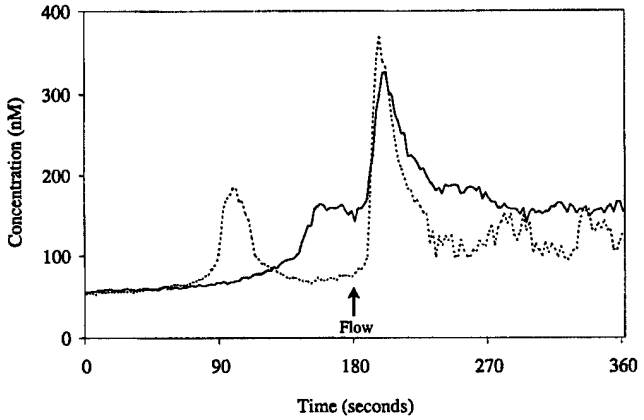


Fig. 6. $[Ca^{2+}]_i$ profiles of 2 individual cells that displayed $[Ca^{2+}]_i$ oscillations in both the baseline and flow periods. Fluid flow was able to induce larger magnitude $[Ca^{2+}]_i$ oscillations in cells that displayed spontaneous oscillations and returned to basal levels before the onset of flow (dashed line) and in cells that had not returned to basal levels before the onset of flow (solid line).

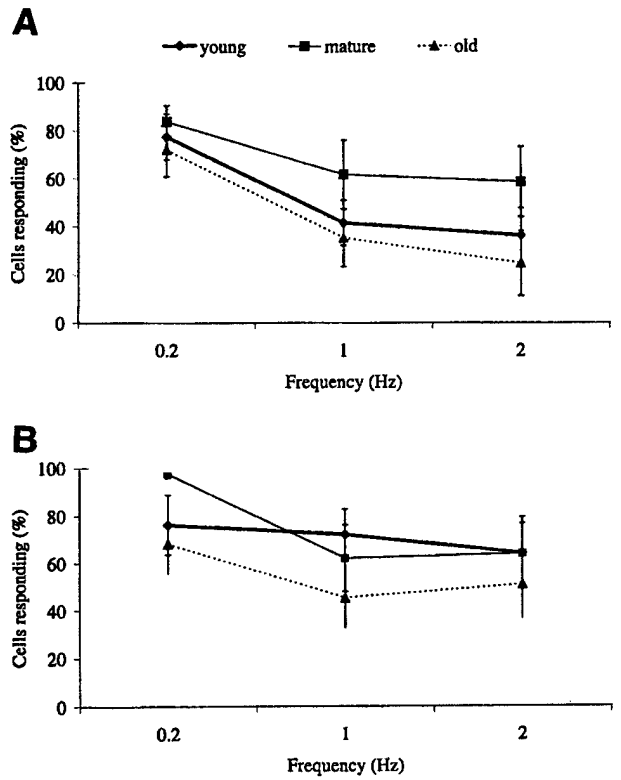


Fig. 7. Influence of age on the percentage of cells displaying $[Ca^{2+}]_i$ oscillations for each flow regime. Values are means with SE bars ($n$ = 7 slides for data point). When each loading regime was considered independently, there were no significant differences among age groups at either 1 (*A*) or 2 Pa (*B*).

pression (5). The spontaneous oscillations that occurred in ROB during the baseline period may have been manifestations of normal cell cycle processes. We found that the number of spontaneous $[Ca^{2+}]_i$ oscillations declined with age in ROB, suggesting that bone cells from old animals are less metabolically active than cells from younger animals. To our knowledge, this is the first demonstration of an age-related decrease in basal $Ca_i^{2+}$ signaling activity in any cell type. Clearly, mechanical stimulation produced a much more abundant and synchronized pattern of $Ca_i^{2+}$ signaling than what occurred in the baseline period. Moreover, the magnitude of the flow-induced responses was significantly larger than the magnitude of the spontaneous oscillations. However, age, loading frequency, and shear stress influenced only the percentage of cells responding to fluid flow; they did not affect the magnitude of the $[Ca^{2+}]_i$ oscillations. These findings suggest that the percentage of cells responding with $[Ca^{2+}]_i$ oscillations may encode physical stimulus information. Indeed, the intercellular propagation of $Ca_i^{2+}$ waves is a mechanism for many cell types to coordinate their activities (21). It also is believed that a threshold of $[Ca^{2+}]_i$ is required to activate a signaling cascade (43). In light of these views, it is reasonable to hypothesize that the abundant higher magnitude $[Ca^{2+}]_i$ oscillations caused by fluid flow encode a different biochemical message than the sparse lower magnitude $[Ca^{2+}]_i$ oscillations that occurred spontaneously during the baseline period.

Aging is known to impair osteoblast differentiation and activity, bone formation, and the material properties of bone (23, 31, 34, 37). Furthermore, aging impairs agonist-stimulated activity of second messengers such as cAMP and $Ca^{2+}$ (14, 24), and $Ca_i^{2+}$ signaling has been linked to the differentiation of cells from the mesenchymal lineage (4). We found that the percentage of cells displaying spontaneous $[Ca^{2+}]_i$ oscillations declined with age; significantly more ROB from young rats displayed spontaneous $[Ca^{2+}]_i$ oscillations than did ROB from old rats. There were also age-related differences in fluid flow-induced $[Ca^{2+}]_i$ oscillations, although in a more complex fashion. Significantly more ROB from mature rats displayed fluid flow-induced $[Ca^{2+}]_i$ oscillations than did ROB from old rats, but there were no differences between young and old ROB. These differences in the age-related trends of basal and fluid flow-induced $Ca_i^{2+}$ signaling are difficult to interpret. Indeed, one would expect that ROB from rapidly growing young rats would display greater responses than ROB from old rats with slower growing bones. However, age-related differences in bone adaptation to unaccustomed mechanical loading has not been evaluated in the three age groups studied here. Although it has been demonstrated that 9-mo-old rats adapt better to mechanical loading than do 19-mo-old rats, it is unclear how the mechanical adaptations of young animals compare with those of mature and old animals (46).

Comparisons with in vivo data are further complicated when the influence of frequency and shear stress on $Ca_i^{2+}$ signaling is examined in ROB. In vivo, bone formation increases when the frequency and magnitude of the mechanical stimulus increases (44, 45). We found contradicting results with $[Ca^{2+}]_i$ oscillations in ROB: the percentage of cells responding increased with increased shear stress but decreased with increased frequency. Clearly there is not a simple relationship between $Ca_i^{2+}$ signaling and bone adaptation. In fact, it is likely that bone adaptation to mechanical loading involves the complex interactions of several mechanotransduction signaling pathways (35). Mounting in vitro mechanotransduction data supports a role for $Ca_i^{2+}$ signaling in bone adaptation to mechanical loading (1, 54). For example, when mechanically induced calcium signaling in bone cells is inhibited by calcium channel blockers, mRNA expression of an abundant bone matrix protein (osteopontin) and the release of a potent stimulator of bone formation (prostaglandin $E_2$) are also inhibited (1, 54). Moreover, calcium channel blockers prevent mechanical loading-induced prostaglandin release in bone organ culture (35). A well-defined role for $Ca_i^{2+}$ signaling in the bone adaptation, mechanotransduction signaling pathway has yet to be elucidated.

Primary bone cell cultures are necessary to study the effects of aging. However, a limitation of ROB cultures is that they likely contain a heterogeneous population of cells derived from bone. We cannot rule out the

possibility that the cultures contain nonosteoblastic or preosteoblastic populations. However, the ROB display an osteoblastic morphology and express phenotypic markers of osteoblasts (i.e., alkaline phosphatase, type I collagen, osteopontin, and parathyroid hormone receptor) (14, 24). It is possible that age-related differences in the degree of cellular heterogeneity of our cell populations contributed to differences in responsiveness to fluid flow. Indeed, this also may be the case in vivo.

There were no differences in the percentage of ROB responding between loading frequencies of 1 and 2 Hz, but a significantly larger percentage of ROB responded to 0.2 Hz. One possible explanation for these findings is cellular viscoelasticity. Because cells are viscoelastic, they may be less stiff and more deformable at lower loading rates. Thus it is possible that the mechanotransducing "machinery" (e.g., stretch-activated ion channels, cell surface receptors, cytoskeleton, etc.) is more likely to be activated at lower loading rates. However, the possibility that the results can be explained, at least in part, by molecular transport phenomena cannot be overlooked. It is well known that serum constituents (e.g., ATP) can function as agonists for $[Ca^{2+}]_i$ oscillations in a concentration-dependent fashion (39, 42, 43). Lower loading frequencies require the mechanical loading apparatus to pump larger volumes of medium, and thus more serum constituents, through the flow chamber to maintain shear stress levels. Therefore, the larger percentage of ROB responding to lower frequency loading may have resulted from larger agonist volumes flowing through the chamber. Similarly, this may explain why 2 Pa were significantly more stimulatory than 1 Pa; the development of higher shear stresses on the flow chamber walls, for a given frequency, requires larger volumes of fluid flow. However, it recently has been shown that shear stress in the absence of serum can stimulate a $Ca_i^{2+}$ signaling in bone cells, but the response is enhanced by mechanical stimulation in the presence of serum (2). Furthermore, it has been shown that parathyroid hormone modulates $Ca_i^{2+}$ signaling in bone cells, suggesting that bone cells may be sensitized to physical stimuli by biomolecules (38). These findings suggest that appropriate levels of both mechanical loading and biochemical constituents are required to mediate cellular mechanotransduction and bone adaptation to mechanical loading.

It has been postulated that gap junctions play a role in communicating mechanical signals in bone cell ensembles (11). In fact, a diverse array of extracellular stimuli (e.g., hormonal, electrical, and mechanical) has been shown to influence gap-junctional intercellular communication, which often involves the propagation of intercellular $[Ca^{2+}]_i$ oscillations (12, 22, 47, 48). However, intercellular $[Ca^{2+}]_i$ oscillations in bone cells also can be propagated by ATP activation of P2Y receptors (22). Because we found no age-related changes in gap-junctional communication in ROB, we postulate that the age-related decrease in the number of cells displaying mechanically induced $[Ca^{2+}]_i$ oscillations involves a defect in the ATP/P2Y mechanism.

In summary, we found that fluid flow-induced $Ca_i^{2+}$ signaling in osteoblastic cells is age, frequency, and magnitude dependent. Cells from young rats showed more basal $[Ca^{2+}]_i$ activity than did old ROB, and mature ROB were more responsive to fluid flow than were old ROB. Low frequency and high shear stress loading regimes were the most stimulatory. We also showed that fluid flow produced higher magnitude and more abundant $[Ca^{2+}]_i$ oscillations than spontaneous oscillations. Ultimately, understanding mechanotransduction pathways in bone cells and how they are influenced by age and mechanical loading parameters may help elucidate the etiologies of bone diseases such as senile and disuse osteoporoses.

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# Temporal aspects of fluid flow induced intracellular calcium oscillations in osteoblastic cells

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**Abstract**

Mechanically driven fluid flow in bone is thought to be an important stimulus for bone cell mechanotransduction and bone adaptation. In vitro, short bouts of fluid flow cause rapid and transient increases in intracellular calcium concentration ($[Ca^{2+}]_i$) in osteoblastic cells. We studied the refractory period for $[Ca^{2+}]_i$ oscillations in primary rat osteoblastic cells during short-term fluid flow and the nature of multiple $[Ca^{2+}]_i$ oscillations during long-term flow. The cells were exposed to 2 minutes of oscillating fluid flow that produced shear stresses of 2 pascals at 2 Hz. After a rest period of 5, 30, 60, 300, 600, 900, 1800, or 2700 seconds, the cells were exposed to a second bout of flow. A 600 second rest period was required to recover the percentage of cells responding to fluid flow and a 900 second rest period was required to recover the $[Ca^{2+}]_i$ oscillation magnitude. The magnitude and shape of multiple $[Ca^{2+}]_i$ oscillations were strikingly similar for individual cells after a 900 second rest period. During 15 minutes of continuous oscillating flow, individual cells displayed between 1 and 9 oscillations subsequent to the initial response. However, only 54 % of the cells that responded initially displayed subsequent $[Ca^{2+}]_i$ oscillations and the magnitude of subsequent oscillations was only 28 % of the initial response. These findings may have important implications for downstream signaling events in osteoblastic cells during long-term fluid flow and for in vivo bone adaptation to mechanical loading.

**Key Words**

Mechanotransduction, osteoblast, calcium signaling, bone adaptation, oscillating fluid flow

## Introduction

Bone adaptation to mechanical loading has been well documented in humans and other animals (N. Ashizawa, et al., 1999; H. Haapasalo, et al., 1996; L. E. Lanyon and C. T. Rubin, 1984; A. G. Robling, et al., 2000; C. T. Rubin, et al., 1995; C. H. Turner, et al., 1994). Currently, the biological mechanisms of bone adaptation are the focus of intense scientific inquiry. Knowledge of the cellular mechanisms involved in mechanically driven bone adaptation will likely underscore pharmaceutical therapies for many bone disorders (e. g., osteoporosis and fracture healing). Mechanically induced fluid flow within the lacuno-canalicular network of bone is believed to be an important bone cell stimulus for mediating bone adaptation to mechanical loading (E. H. Burger and J. Klein-Nulend, 1999; S. C. Cowin, et al., 1995; S. Weinbaum, et al., 1994). Fluid flow provides a mechanism for the transport of nutrients and waste products; it can also provide individual bone cells with information about the mechanical forces acting on whole bones. In vitro, bone cells respond to physical stimuli with a cascade of biological signaling events by a process known as mechanotransduction. One of the earliest events in bone cell mechanotransduction is intracellular calcium signaling. Fluid flow, which engenders cell membrane shear stress, induces a rapid and transient increase in intracellular calcium concentration ($[Ca^{2+}]_i$) in osteoblastic cells (F. D. Allen, et al., 2000; N. X. Chen, et al., 2000; C. T. Hung, et al., 1995; C. R. Jacobs, et al., 1998). Mechanically induced $[Ca^{2+}]_i$ oscillations have been shown to influence downstream events such as gene expression. Inhibiting $[Ca^{2+}]_i$ oscillations in osteoblastic cells inhibited fluid flow induced mRNA upregulation of the bone matrix protein osteopontin (J. You, et al., 2001).

In addition to influencing gene expression, $Ca^{2+}$ signaling is implicated in numerous other cellular activities (e. g., proliferation, differentiation, and apoptosis) in many cell types (M.

J. Berridge, et al., 1998). However, the mechanisms for the pleiotropic actions of $Ca^{2+}$ are only partially understood. The magnitude, duration, and frequency of $[Ca^{2+}]_i$ oscillations are all believed to play a role in regulating downstream events (A. P. Thomas, et al., 1996; E. C. Toescu, 1995). For example, varying the frequency of the $[Ca^{2+}]_i$ oscillations may be used to activate different genes (M. J. Berridge, et al., 1998), and the enzyme CaM Kinase II has been shown to effectively "count" $[Ca^{2+}]_i$ oscillations and vary its activity accordingly (P. De Koninck and H. Schulman, 1998). Agonists, which stimulate the release of calcium from the intracellular stores of smooth muscle cells, can induce $[Ca^{2+}]_i$ oscillations of 400 to 800 nM; the $[Ca^{2+}]_i$ oscillation frequency is dose-dependent, ranging between 4-30 oscillations per minute (J. P. Savineau and R. Marthan, 2000). Osteoblastic cells typically display a single $[Ca^{2+}]_i$ oscillation when exposed to short bouts (1.5 - 3 minutes) of fluid flow (C. T. Hung, et al., 1996; C. T. Hung, et al., 1995; C. R. Jacobs, et al., 1998). However, multiple oscillations in some cells have been noted, but not characterized (C. T. Hung, et al., 1995).

The ability of ensembles of osteoblastic cells to respond to multiple bouts of fluid flow, separated by a 10 – 15 minute rest period, has been noted previously (C. T. Hung, et al., 1995). It was found that 58% of the cells responded to the initial loading bout and 45% responded to the second. The effect of rest periods, of varying durations, on $Ca^{2+}$ signaling in osteoblastic cells has not been studied. Additionally, $Ca^{2+}$ signaling in osteoblastic cells exposed to continuous loading of durations longer than 3 minutes has also not been studied previously. Yet, many in vitro mechanotransduction experiments use long-term (on the order of hours) bouts of fluid flow to study other biochemical responses such as prostaglandin release or gene expression (N. E. Ajubi, et al., 1999; J. You, et al., 2001). Since $Ca^{2+}$ signaling is linked to the upregulation of

gene expression and prostaglandin release during long-term fluid flow, it will be important to elucidate the temporal aspects of $Ca^{2+}$ signaling during long-term flow.

An understanding of the temporal aspects of $Ca^{2+}$ signaling is also important for establishing relationships between in vitro mechanotransduction studies and in vivo adaptation phenomena. Histological changes in the architecture of bone have been characterized in animals exposed to unaccustomed mechanical loading. Partitioning a daily mechanical stimulus into discrete loading bouts enhances bone formation in rat tibiae (A. G. Robling, et al., 2000). This finding suggests that the bone cells involved in mediating mechanically induced bone adaptation have a refractory period, during which they are insensitive to additional mechanical stimuli. Bone formation was enhanced when 360 loading cycles were divided up into distinct bouts spaced over the course of a day, compared to a single bout of 360 cycles (A. G. Robling, et al., 2000). Based on these findings, we hypothesized that 1) a refractory period exists, during which time fluid flow induced $[Ca^{2+}]_i$ oscillations in bone cells are insensitive to additional bouts of fluid flow; and 2) that longer-term (15 minutes) continuous fluid flow produces multiple $[Ca^{2+}]_i$ oscillations in osteoblastic cells.

**Method**

*Bone Cells*

Rat osteoblastic cells (ROB) were isolated from the humeri, tibiae, and femora of 4-month-old male Fisher 344 rats. All procedures were approved by the Institutional Animal Care and Use Committee at the M. S. Hershey Medical Center. The rats were anesthetized by an intraperitonial injection of pentobarbital sodium (Nembutal, Abbott Laboratories, North Chicago, IL) with a dosage of 50 mg/Kg of bodyweight and euthanized by exsanguination. The bones were extracted from the animals and subperiosteal ROB were obtained by removing all soft

tissues, including cartilage and periosteum, from the bones and performing sequential collagenase (Worthington Biochemical Corporation, Lakewood, NJ) digestions at 37°C. Cells from the first digestion were collected by centrifugation and discarded to eliminate any residual non-bone cells that were not removed by dissection. Cells from the second digestion were collected by centrifugation and grown to confluency in Dulbecco' Modified Eagle Medium (DMEM, Gibco, Gaithersburg, MD), 20% fetal bovine serum (FBS), and 1% penicillin/streptomycin. We have previously shown that ROB isolated by this technique display characteristics of the osteoblast phenotype (H. J. Donahue, et al., 1995). Three days prior to experimentation the cells were plated on quartz microscope slides (76 mm × 26 mm × 1.6 mm) at a density of 75,000 cells per slide; cells were approximately 80% confluent on the day of experimentation. The cells were incubated at 37°C with 10 μM Fura-2 AM (Molecular Probes, Eugene, OR) for 30 minutes prior to mechanical stimulation.

*Fluid Flow System*

Following Fura-2 loading the cell-seeded microscope slides were mounted in a parallel plate flow chamber, which was fixed to the stage of a fluorescent microscope. A fresh bolus of flow media was added to the chamber and the cells were left undisturbed for 30 minutes. The flow media consisted of DMEM and 2% FBS. We used a previously described fluid flow system to expose ROB to oscillating fluid flow (C. R. Jacobs, et al., 1998). To generate fluid flow induced shear stresses on the cells in the chamber, a materials testing machine was used to pump a syringe, which was in series with rigid wall tubing and a flow meter (Transonic Systems Inc., Ithaca, NY), driving fluid through the chamber. This system produces laminar fluid flow in the chamber with an oscillating flow profile. Shear stresses on the chamber walls are dependent on the chamber dimensions and the rate of fluid flow (C. T. Hung, et al., 1995). Thus, we were able

to generate shear stresses on the cells with magnitudes that they are predicted to experience *in vivo* (S. Weinbaum, et al., 1994). Oscillating fluid flow was used because it more closely simulates physiologic bone loading than steady or pulsatile flow (C. R. Jacobs, et al., 1998). During experimentation the cells were exposed to 2 minutes of oscillating fluid flow that produced shear stresses of 2 pascals (Pa) at a frequency of 2 Hz. After a rest period of 5, 30, 60, 300, 600, 900, 1800, or 2700 seconds, the cells were exposed to a second bout of fluid flow. In a second set of experiments, different groups of cells were exposed to 15 minutes of continuous oscillating flow.

*Calcium Imaging*

Real-time $[Ca^{2+}]_i$ was quantified using ratiometric dye methodology. When Fura-2 binds $Ca^{2+}$, its maximal absorption wavelength shifts from 363 nm for $Ca^{2+}$-free Fura-2 to 335 nm for $Ca^{2+}$-bound Fura-2 (A. Takahashi, et al., 1999). In practice, wavelengths of 340 and 380 nm are used for ratiometric measurements. The emission peak is near 510 nm for both $Ca^{2+}$-free and $Ca^{2+}$-bound Fura-2. ROB cell ensembles were illuminated at wavelengths of 340 and 380 nm; emitted light was passed through a 510 nm filter and images were collected with a CCD camera. Images of fluorescence intensities were collected every two seconds for a one minute no flow period (baseline) and during the 2 minute bouts of oscillating fluid flow. $[Ca^{2+}]_i$ was determined from the ratio of the two emission intensities using calibrated standards and image analysis software (Metaflour, West Chester, PA). Temporal $[Ca^{2+}]_i$ profiles were determined for at least 50 individual cells for each rest period. We defined a responsive cell as one that displayed a transient increase in $[Ca^{2+}]_i$ of at least 4-fold the maximum oscillation value recorded during the baseline period. We assessed the percentage of cells responding with a $[Ca^{2+}]_i$ oscillation and the magnitude of the $[Ca^{2+}]_i$ oscillations.

*Statistics*

One-way ANOVA's were used to look for differences, between the first and second loading bouts, in the percentage of cell responding and the magnitude of the response for each rest period using Statview software (SAS Institute, San Francisco, CA). Because of heterogeneity in $[Ca^{2+}]_i$ responses, the magnitude of the $[Ca^{2+}]_i$ oscillations and the percentage of cells responding are presented graphically as the ratio of the oscillation that occurred during the second bout of fluid flow to the oscillation that occurred during the first bout of fluid flow to facilitate comparisons between rest period groups. For the continuous loading regime, ANOVA's were used to compare the percentage of cell responding and the magnitude of the response between the first and subsequent oscillations. A significance level of 0.05 was used for all statistical analyses.

**Results**

With the onset of the first bout of fluid flow there were rapid and transient increases in $[Ca^{2+}]_i$, which lasted approximately 60 seconds (Fig. 1). Peak values were reached approximately 15 seconds after the onset of fluid flow. During the first bout of fluid flow, the mean (± SE) percentage of cells displaying $[Ca^{2+}]_i$ oscillations was 96.7 ± 1.3 % with a mean (± SE) magnitude of 185 ± 6 nanomolar (nM). Some cells could respond to a second bout of fluid flow after only a 5 second rest period; however, only 20 % of the cells that responded during the first bout could respond during the second bout (Fig. 2). The percentage of cells responding to the second bout of flow was significantly ($p < 0.049$) lower than the percentage responding to the first bout for rest periods less than 10 minutes (Fig. 2). For rest periods of 10 minutes or longer there were no significant ($p > 0.095$) differences in the percentage of cells responding between the two bouts of fluid flow.

Although some cells could respond to the second bout of flow after only a 5 second rest period, the magnitude of the $[Ca^{2+}]_i$ oscillation during the second bout of fluid flow was only 36 % of the oscillation magnitude during the first bout of flow (Fig. 3). The magnitude of the second $[Ca^{2+}]_i$ oscillation was significantly ($p < 0.008$) lower than the magnitude of the first $[Ca^{2+}]_i$ oscillation for rest periods less than 15 minutes (Fig. 3). After a 15 minute rest period, 94 $\pm$ 2 % of the cells that responded to the first bout of flow responded to the second bout with 89 $\pm$ 3 % of the magnitude of the first $[Ca^{2+}]_i$ oscillation. Thus, a 15 minute rest period between loading bouts was required to regain both the percentage of cells responding and the magnitude of the $[Ca^{2+}]_i$ oscillations.

Not only was there no significant difference in the oscillation magnitude after at least a 15 minute rest period, there was a striking similarity in the multiple $[Ca^{2+}]_i$ oscillation profiles of individual cells. If individual cells responded to the first bout of fluid flow, they typically responded to the second bout with the same magnitude $[Ca^{2+}]_i$ oscillation (Fig. 4). If cells did not respond to the first loading bout, they typically did not respond to the second (Fig. 4). Furthermore, not only was there a striking similarity in the $[Ca^{2+}]_i$ oscillation magnitudes for multiple oscillations in individual cells, there were remarkable similarities in the shape and duration of the $[Ca^{2+}]_i$ oscillation profiles (Fig. 5).

During 15 minutes of continuous oscillating fluid flow many cells displayed multiple $[Ca^{2+}]_i$ oscillations. Individual cells displayed between 1 and 9 $[Ca^{2+}]_i$ oscillations subsequent to the initial response (Figs. 6a and b), with a mean of 3.9 $\pm$ 0.5 subsequent oscillations. 100 % of the cells exposed to 15 minutes of continuous oscillating fluid flow displayed an initial response. However, only 54 % of the cells that responded initially displayed subsequent $[Ca^{2+}]_i$ oscillations

(Fig. 7). Furthermore, the magnitude of the subsequent $[Ca^{2+}]_i$ oscillations were only 28 % of the initial response (Fig. 8).

**Discussion**

While it is clear that bones adapt to their mechanical loading environment, it is unclear how bone cells accomplish adaptation phenomena. In vitro, bone cells respond to physical stimuli with numerous biochemical responses. However, the details of the mechanotransduction signaling pathways are only beginning to emerge. It is well established that $Ca^{2+}{}_i$ signaling in osteoblastic cells is one of the earliest responses to fluid flow (F. D. Allen, et al., 2000; N. X. Chen, et al., 2000; C. T. Hung, et al., 1996; C. T. Hung, et al., 1995; C. R. Jacobs, et al., 1998). It has also been demonstrated that $Ca^{2+}{}_i$ signaling is required for downstream events such as the upregulation of osteopontin mRNA during long-term fluid flow.(J. You, et al., 2001) However, the nature of the temporal aspects of $Ca^{2+}{}_i$ signaling during long-term flow and how they mediate other components of the signaling cascade are unclear. Since bone cells engender whole bone adaptations to mechanical loading it is likely that some, if not all, of the components of the mechanotransduction signaling cascade have similar temporal characteristics as mechanically induced bone formation in whole bones. When a mechanical stimulus was partitioned into distinct loading bouts, periosteal bone formation was enhanced in rat tibiae, suggesting the existence of a refractory period in the mechanotransduction signaling cascade of bone cells (A. G. Robling, et al., 2000). Therefore, we studied the refractory period for $[Ca^{2+}]_i$ oscillations in bone cells exposed to distinct bouts of fluid flow and the nature of multiple $[Ca^{2+}]_i$ oscillations during continuous long-term flow.

$[Ca^{2+}]_i$ oscillations are associated with the activation of numerous $Ca^{2+}{}_i$-dependent enzymes which are involved in many different cellular functions (M. J. Berridge, et al., 1998).

With the onset of a $[Ca^{2+}]_i$ oscillation there is also an immediate activation of processes that restore $[Ca^{2+}]_i$ to resting levels to prevent extended exposure to toxic levels of $[Ca^{2+}]_i$ (E. C. Toescu, 1995). During the $[Ca^{2+}]_i$ oscillation, cytosolic $Ca^{2+}$ is rapidly buffered by many different molecules such as nucleotides, organic acids, and proteins, which alters the diffusion coefficient of cytosolic $Ca^{2+}$ (E. C. Toescu, 1995). Cytosolic $Ca^{2+}$ is also pumped out of the cell and back into intracellular stores by $Ca^{2+}$-ATPases. The rates of buffered $Ca^{2+}$ diffusion in the cytosol and the kinetics of refilling intracellular $Ca^{2+}$ stores may determine the refractory period for fluid flow induced $[Ca^{2+}]_i$ oscillations. We found that a 10 minute rest period was required to recover the percentage of cells responding to a bout of fluid flow and a 15 minute rest period was required to recover the magnitude of the $[Ca^{2+}]_i$ oscillations. These periods may represent the time intracellular $Ca^{2+}$ stores need to regain their homeostatic $[Ca^{2+}]$ so that they are ready for another signaling event.

Hung et al. (1995) found that 58% of cells responded to an initial bout of fluid flow and 45% responded to a second bout after a 10-15 minute rest period, but most of the cells responding to the second bout had not responded to the first bout. This is inconsistent with our finding that cells which displayed oscillations typically did so in both flow periods for rest periods of 10 minutes or longer. There are many possible explanations for this discrepancy. For example, they used primary cells isolated from neonatal rat calvaria and a serum-free perfusate with a steady flow profile. Indeed, it has been demonstrated that cells display differential calcium responses to oscillating and steady flow (C. R. Jacobs, et al., 1998). The results of Hung et al. (1995) may be viewed as the refractory nature of a pure mechanotransduction response to steady flow because of the absence of serum. Whereas our findings may reflect a chemotransduction response or an integrated chemo/mechanotransduction response to oscillating

flow. An important note on our data is worth consideration as it represents a limitation in determining cellular responses for the shorter rest periods (i. e., $\leq$ 1 minute). Immediately following the return of the initial $[Ca^{2+}]_i$ oscillations to baseline values, the $[Ca^{2+}]_i$ profiles displayed more noise or low magnitude fluctuations than they did during the initial baseline period (fig 1). Therefore the cells that were quantified as responders to the second bout of flow, for rest periods of 1 minute or less, may have actually still been fluctuating due to the initial bout rather than responding, per se, to the second bout. In that case the values of the percentage of cells responding and the magnitude of the response that were presented for these short rest periods may actually be even smaller.

The heterogeneity of $[Ca^{2+}]_i$ responses to fluid flow and agonist stimulation has been previously noted.(R. Civitelli, 1992; C. T. Hung, et al., 1995; C. R. Jacobs, et al., 1998) Individual cells display unique combinations of $[Ca^{2+}]_i$ oscillation characteristics such as the magnitude, rise and fall times, duration, and overall shape. These characteristics define what has been termed a "$Ca^{2+}$ fingerprint" (M. Prentki, et al., 1988). The unique $[Ca^{2+}]_i$ oscillation profiles of individual cells, stimulated by the agonist carbamylcholine, were almost identically reproduced when the cells were restimulated carbamylcholine (M. Prentki, et al., 1988). Hung et al. (1995) proposed that shear stress gradients may contribute to the heterogeneity of fluid flow induced $[Ca^{2+}]_i$ oscillations. However, the addition of the calcium agonist bradykinin, in the absence of flow, also produced a heterogeneous response (C. T. Hung, et al., 1995). Prentki et al. (1988) suggested that the $Ca^{2+}$ fingerprint is an intrinsic property of individual cells, which is independent of stochastic events and allows them to respond in a selective and reproducible manner. Indeed, the reproducible profiles of multiple $[Ca^{2+}]_i$ oscillations in individual

osteoblastic cells, exposed to multiple bouts of fluid flow (fig 5), suggest that $Ca^{2+}$ fingerprints exist in osteoblastic cells and may have a role in mechanotransduction.

Bone cells typically display a single $[Ca^{2+}]_i$ oscillation when exposed to short bouts (1.5 - 3 minutes) of steady, pulsatile, or oscillating fluid flow.(C. T. Hung, et al., 1996; C. T. Hung, et al., 1995; C. R. Jacobs, et al., 1998) However, multiple oscillations have been noted during short bouts of flow (C. T. Hung, et al., 1995). We found that during 15 minutes of continuous oscillating fluid flow, distinct $[Ca^{2+}]_i$ oscillations could occur as shortly as 60 seconds after the initial $[Ca^{2+}]_i$ oscillations had returned to baseline values. As many as 9 subsequent $[Ca^{2+}]_i$ oscillations were observed in individual bone cells during the 15 minute loading period. However, the percentage of cells displaying subsequent oscillations and the magnitude of the subsequent $[Ca^{2+}]_i$ oscillations were significantly lower than the initial $[Ca^{2+}]_i$ responses. It is possible that the subsequent low magnitude and low frequency $[Ca^{2+}]_i$ oscillations encode a different biochemical message than the initial response. Indeed, low frequency $[Ca^{2+}]_i$ oscillations may be integrated into a potent biological message over time, such as incremental $Ca^{2+}$-dependent phosphorylation of regulatory proteins (J. W. Putney, Jr. and G. S. Bird, 1993). The finding that some cells display multiple $[Ca^{2+}]_i$ oscillations during long-term oscillating flow may have important implications for downstream events such as the upregulation of gene expression. For example, two hours of continuous oscillating fluid flow increased osteopontin gene expression 4-fold over no flow control levels; however, this increase was prevented when cells were treated with the $Ca^{2+}$-ATPase inhibitor thapsigargin to prevent the release of $Ca^{2+}$ from intracellular stores (J. You, et al., 2001). It is reasonable to hypothesize that multiple $[Ca^{2+}]_i$ oscillations during long-term flow influence gene expression by acting on $Ca^{2+}$-dependent enzymes such as CaM Kinase II.

Clearly bone cells display two distinct calcium responses to fluid flow. The first is the immediate response that occurs with the onset of flow, in which individual cells display large heterogeneity in the $[Ca^{2+}]_i$ oscillation magnitude, but relative homogeneity in the time to activation and duration of the $[Ca^{2+}]_i$ oscillation. This response can be reproduced, with striking similarity in the $[Ca^{2+}]_i$ profiles of individual cells, when rest periods greater than 15 minutes are inserted between loading bouts. The second type of response is the multiple $[Ca^{2+}]_i$ oscillations that occur subsequent to the initial response during long-term fluid flow. These subsequent $[Ca^{2+}]_i$ oscillations have lower magnitudes than the initial responses and display large heterogeneity in the number of subsequent oscillations that occur in individual cells as well as in the oscillation magnitude. Our findings raise the possibility that multiple, low amplitude $[Ca^{2+}]_i$ oscillations are involved in regulating the downstream responses of bone cells to long-term fluid flow such as gene expression. Partitioning a daily mechanical stimulus into discrete loading bouts enhances bone formation in rat tibiae (A. G. Robling, et al., 2000). Our finding that the magnitude of the $[Ca^{2+}]_i$ oscillation and the percentage of cells responding could be regained, when rest periods of at least 15 minutes were inserted between loading bouts, supports a role for $Ca^{2+}$ signaling in bone adaptation to mechanical loading. For cell processes where the oscillation magnitude and percentage of cells responding are important, rest periods may increase the number of times those processes are activated.

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